TECHNIQUES OF
HISTO- AND CYTOCHEMISTRY

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A Manual of Morphological and Quantitative Micromethods for Inorganic, Organic and Enzyme Constituents in Biological Materials

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This Book is Dedicated to

KAJ LINDERSTRØM-LANG and HEINZ HOLTER

of the Carlsberg Laboratory, Copenhagen, in appreciation of their scientific achievements, which have contributed generously to the development of histo- and cytochemistry, and in appreciation of their fine human qualities of integrity, understanding, and a truly civilized sense of values and of humor.

FOREWORD

To the older biologist, microchemistry meant the application of appropriate reagents to sections of tissue or to intact cells to enable him to recognize under the microscope the nature and localization of compounds in living substance. To the chemist, on the other hand, it signifies the application of instruments of great precision and rigorous methods to the accurate determination of the composition of extremely small amounts of material. To the modern biologist, it includes all of those methods which may aid him to delineate in the exiguous confines of the cell that elusive and mysterious chemical pattern which is the basis of life. To the extent that it requires isolation and purification of compounds, microchemistry is but a significant extension of the usual methods of biochemistry, but with the discovery of methods of isolating microscopic and submicroscopic and even ultramicroscopic components of living substance, and the application of physical and chemical methods of analysis to them, the new microchemistry promises to become the most important tool we possess for elucidation of the fundamental chemical pattern of protoplasm.

In our enthusiasm for these methods, however, we must not forget that by far the most sensitive instrument for microchemical analysis is the living organism itself. The methods of immunology, for example, suffice to discriminate between compounds so closely related that the chemist is at a loss to distinguish between them. The genes revealed by genetic experiment exceed by an infinite multiple the meager number of nucleoproteins revealed by biochemical research. The bioassay methods depend on the exquisite sensitivity of the living organism to minute changes in its chemical environment. These are also microchemical methods.

In this volume, the author has chosen to follow the historical pattern and to present the methods of microscopical analysis first. In this field, the difficulty is not so much to find suitable reagents as to prepare material in a form susceptible to microscopic study. Botanical material for a long time possessed definite advantages, since the support afforded by cellulose walls, and, in higher plants,

by the vascular bundles, enabled the worker to obtain sections without previous preparation. The introduction of the freezing-drying technique by Gersh removed to some extent this advantage and enabled the worker to obtain sections of animal material without previous chemical treatment. This valuable method has been wisely chosen as the first subject of the first section.

This section is devoted to the methods for recognition of chemical substances in microscopic preparations. The author has contented himself with presenting accurately and without prejudice the many methods so far suggested for the detection of various chemical substances in tissues. In this field there are three purposes to be served —namely, recognition, localization, and quantitation. The first of these may be accomplished equally well by macrochemical methods and the third has only recently achieved importance through the introduction of the newer physical methods of measurement. Accordingly, localization becomes the chief function of microchemistry of this order. On the user rests the responsibility for perceiving and avoiding the many pitfalls which are inevitable. Gross blunders have been made in the past and can only be avoided in the future by meticulous care and high critical ability on the part of the worker. The belief that in the complex colloidal matrix of protoplasm reactions occur as they do in more simplified systems *in vitro* is responsible for many mistakes. No assumption as to solubility or insolubility is admissible. Otherwise soluble substances may be firmly adsorbed to submicroscopic surfaces or incorporated into molecular aggregates. In those methods which require fixing, imbedding, and cutting in order to prepare the material for microchemical tests, the disparity between the mass of material and the volume and variety of solvents employed makes the data on insolubility equally untrustworthy. The worker must examine critically each step of the process and seek to appraise its effect on the final result. Reaction time, drift of highly dispersed reaction products to other locations due to surface charges, and translocation of reaction owing to differences of ion mobility, all must be carefully considered.

Microchemical reactions yielding colorless products and requiring the use of a second reagent to visualize them should be accepted only with reservation and should be used only as a first approach, subject to the results of later confirmatory tests. To this category belong, for example, the molybdate reactions for the detection of phosphate,

Macallum's reaction for potassium, and the popular methods for the detection of phosphatases. In all of these, the possibility for the adsorption of molybdic, cobalt, or lead ions, respectively, independent of the reactions supposed to occur, should be entertained.

Negative results should not be accepted; the limitations imposed by the microscope as to the thickness of preparations both from the standpoint of transparency and of dispersion of light make any negative conclusions inadmissible.

It may be inferred from the preceding remarks that this writer views microchemical methods of this category with suspicion. This is not the case. He simply wishes to insist that the worker scrutinize critically every phase of his technique and consider seriously what the value of the method may be for recognition, quantitation, and localization, respectively.

The second, third, and fourth parts of the volume are devoted to the methods for accurate physical and chemical microanalysis as applied to biological problems, and to the newer methods for the mechanical separation of the morphological constituents of protoplasm. This writer has already indicated above his belief that in these methods rests our chief hope of progress in the solution of the mystery of life.

The road to this goal is a long and laborious one. It is the writer's hope and belief that travelers along this difficult highway will find their burdens lightened by the collection into one volume of so many useful methods of investigation.

R. R. Bensley

PREFACE

"But not by nature is the man of science more critical or careful than his colleagues are. What gives him an advantage over them is that when issues rise in his domain they can be settled with a sureness and dispatch which elsewhere are unknown; for science has a priceless touchstone here to seek out truth—the technique of measurement."
EDMUND W. SINNOTT in *Science and the Education of Free Men,* American Scientist 32: 209 (1944).

Like a lens gathering diverse rays and concentrating them in a new beam which penetrates depths hitherto unilluminated, each new border science reveals a new threshold of knowledge. We who work in the life sciences stand at such a threshold today. The lights of histology and cytology are being joined with those of chemistry, and the brilliant new beams converging to a focus probe beyond the old limitations. The day is past when our vision cannot penetrate beyond the architecture of the cell. The wealth of knowledge that has been, and can still be gleaned from purely descriptive microscopic anatomy is not to be minimized, but under the new illuminations we can begin to discern the chemical patterns in the cellular architecture. From this knowledge an understanding of the functions of the patterns will follow. Thus, from histology and cytology, as we have known them in the past, the new field of histo- and cyto-

chemistry is arising, and from this new field, a histo- and cyto-physiology will develop—and so on in the expanding and exciting quest into the nature of living processes. These new instruments and techniques which carry our vision deep into the living unit, to the molecules and the atoms—they include the beautiful ingenuities which have been refined out of the mountain of past scientific experience, and some which have been newly created for the purpose. It is these instruments and techniques with which we shall be concerned in this book.

In a discussion on cytological technique, J. R. Baker (1942) of Oxford stated, "It was once remarked to the writer that biochemists like to have their substances in test-tubes. The cytologist wants to have his exactly where they were in life, and to know, as precisely as he can, what they are. When substance and structure are known, the way is clear for the elucidation of the main problem of cytology, which is to discover what a cell does to keep alive and to perform its functions for the body as a whole or for the next generation." It isn't so much "that biochemists like to have their substances in test-tubes" as it is that, until not long ago, biochemists had no means of dealing with substances except in "test-tubes." That biochemists have been fundamentally dissatisfied with this limitation is apparent in their growing efforts to refine their techniques to enable investigations *in situ*. We can be sure that future campaigns designed to assault the present horizons of cytology will follow the strategic lines made possible by the development of equipment and procedures which bring chemical investigations directly to the cell, and the parts of the cell, existing in natural milieu. In the following pages we shall examine the techniques and devices already elaborated for this purpose, and no one with imagination will fail to be impressed and excited by the possibilities engendered.

Lison's *Histochemie Animale*, a book dealing mainly with microscopic techniques of chemical morphology, was published in Paris in 1936. Many notable advances have occurred since then, and the present volume has been designed to bring together in a compact and readily available form detailed descriptions, not only of the morphologic, but also of the quantitative techniques. Insofar as it has been possible, the material presented has been brought up to date as of January 1, 1947. Pertinent publications which have appeared between January 1 and September, 1947, have not been

discussed, but a bibliography appendix containing these references has been included at the end.

To those who treat with condescension all science that cannot be quantitated, it might be said, "True, morphology is only a stepping stone, and while as a stepping stone it is not a place to stop, it is none the less basic." And to those whose comprehension is confined to mere morphology, it might be said, "True, morphology is basic, but it is only a stepping stone in science." And to both it should be said, "All fields of science are stepping stones, and in science there are no places to stop."

The author wishes to express his appreciation to the following for critically reviewing various sections of this volume and offering many helpful suggestions: Dr. R. R. Bensley, Dept. of Anatomy, University of Chicago; Dr. W. L. Doyle, Dept. of Anatomy, University of Chicago; Dr. O. H. Lowry, Dept. of Pharmacology, Washington University; Dr. G. H. Scott, Dept. of Anatomy, Wayne University; and Dr. J. M. Tobias, Dept. of Physiology, University of Chicago. The encouragement in this undertaking of Dr. M. B. Visscher, Dept. of Physiology, University of Minnesota; and Dr. B. Sullivan, Director of Laboratories, Russell-Miller Milling Co., Minneapolis, is also gratefully acknowledged. The author is grateful for the invaluable assistance of the publisher's staff.

DAVID GLICK

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ABBREVIATIONS

l.	liter	ft.	foot
ml.	10^{-3} liter	in.	inch
μl.	10^{-6} liter	yr.	year
gal.	gallon	hr.	hour
g.	gram	min.	minute
mg.	10^{-3} gram	sec.	second
μg.	10^{-6} gram	amp.	ampere
mμg.	10^{-9} gram	μamp.	10^{-6} ampere
lb.	pound	μF.	10^{-6} farad
oz.	ounce	Kev	10^{3} electron volts
D.C.	direct current	M	molar
R.P.M.	revolutions per minute	mM	10^{-3} molar
E.M.F.	electromotive force	N	normal
m.	meter	conc.	concentrated
cm.	10^{-2} meter	dil.	dilute
mm.	10^{-3} meter	soln.	solution
μ	10^{-6} meter	sp. gr	specific gravity
mμ.	10^{-9} meter	C. P.	chemically pure
Å	10^{-10} meter (angstrom unit)	c.p.	candle power
		m.p.	melting point
X.U.	10^{-3} angstrom	b.p.	boiling point

All temperatures are given in degrees centigrade.

If not otherwise indicated, all solutions are understood to be aqueous.

If not otherwise indicated, the term *alcohol* refers to 95% ethyl alcohol.

MICROSCOPIC TECHNIQUES

"She (Science) warns me to be careful how I adopt a view which jumps with my preconceptions, and to require stronger evidence for such a belief than for one to which I was previously hostile. My business is to teach my aspirations to conform themselves to fact, not to try and make facts harmonize with my aspirations.—Sit down before fact as a little child, be prepared to give up every preconceived notion, follow humbly wherever and to whatever abysses nature leads, or you shall learn nothing."

THOMAS HUXLEY
in a letter to Charles Kingsley, September 23, 1860.

The microscopic techniques which are treated in the present volume are those designed to establish the distribution of elements, groups, substances or activities in microtome sections of tissue by means of examinations under some form of microscope. This requires that the factor in question be made apparent by characteristic optical or photographic properties such as a specific color, fluorescence, or radiation. With the exception of absorption histospectroscopy, these microscopic techniques are limited to observations which are essentially qualitative in nature. However, the microscopic techniques permit a much greater degree of localization of particular chemical constituents in histologically defined cells, or cytologically defined parts of cells, than is possible by means of the quantitative chemical techniques. Thus, one is often forced to choose between degree of localization and quantitation. Obviously, it would be preferable to establish both the cellular disposition of biologically significant factors and their quantitative relationships.

I. FREEZING-DRYING PREPARATION OF TISSUE

Since almost all of the microscopic techniques that will be discussed are based on the use of microtome sections of tissue, it is pertinent that the freezing-drying preparation for sectioning be described in detail. This technique of sudden cooling to low temperatures and rapid dehydration of the frozen material *in vacuo* has many advantages over the usual histological methods employing fixing and dehydrating solutions. The chief of these advantages are a minimum of chemical change in the tissue (there is an almost instantaneous cessation of metabolic activity and no chance for other chemical changes to occur), a minimum of shifting of diffusible constituents (fluid is not used and the fixation is immediate), a greater preservation of cytoplasmic inclusions than is possible with the use of fixing solutions, the possibility of direct paraffin infiltration of de-

hydrated tissue, and the absence of cell shrinkage. These are no inconsiderable advantages, and the freezing-drying technique should be given the preference wherever possible.

Scott (1943) has been careful to point out that, while distortion of mineral distribution might be expected to occur as the result of ice crystal formation during the freezing and that artifacts might be occasioned by the paraffin infiltration, neither of these appears to be a serious difficulty in the more recent improved techniques. As Scott indicated, on the one hand ice crystal formation could be readily recognized should it occur in a manner that might influence interpretation, and on the other it has been impossible thus far to find evidence of distortion resulting from the infiltration, although various control experiments have been performed to test this possibility.

Gersh (1932) extended the Altmann method of dehydrating tissue *in vacuo* at liquid-air temperatures, and the improved procedure is known as the Altmann-Gersh technique.* In a critical study Scott (1933a) pointed out that the dehydration temperature of —20° used by Gersh was not low enough to prevent a certain amount of ion diffusion since this temperature is above the eutectic point of certain naturally occurring salt systems. In the improved cryostat of Packer and Scott (1942), to be described in detail later, the temperature is maintained below —54.9°, the eutectic point of $CaCl_2.6H_2O$. When he first indicated the desirability of using lower temperatures, Scott (1933a) recommended the use of alcohol cooled to —177°, instead of liquid air, since the latter gives rise to a gas envelope around the tissue which retards the rate of freezing. A further improvement was effected by Hoerr (1936), who found that more rapid freezing (hence smaller ice crystals) was obtained by placing tissue in isopentane cooled to —160° to —195° by means of liquid nitrogen. Among others, Simpson (1941) confirmed the advantages of the isopentane method and, in addition, pointed out the desirability of employing small pieces of tissue for treatment since the centers of larger pieces do not yield sections of the highest quality.

After the appearance of the Gersh (1932) vacuum dehydrator, other types were described by Goodspeed and Uber (1934) and Scott and Williams (1936). However, since none of these was

* The Gersh apparatus is available from *A. S. Aloe & Co.*

wholly satisfactory, Packer and Scott (1942) developed a cryostat of a new design that is the finest instrument yet devised for the freezing-drying of tissues. An important feature of this apparatus is that the frozen and thoroughly dried tissue can be brought gradually to the temperature of the melted paraffin, and then it can be embedded without contact with the moisture of the air. Previous practice was to transfer the very cold tissue from the cryostat to the air, and then plunge it directly into melted paraffin, thus subjecting it to a sudden temperature change of about 100°. Sjöstrand (1944) described a freezing-drying apparatus somewhat simpler than the Packer-Scott instrument but it was not designed to permit paraffin infiltration within the apparatus.

PACKER-SCOTT METHOD FOR FREEZING-DRYING TISSUES

The Freezing-Drying Apparatus. In the diagram of the apparatus, which is made of Pyrex glass (Fig. 1), the drying chamber (*C*) is a 2.5 in. tube 12.5 in. long surrounded by a jacket of about 3.5 in. diameter that can be exhausted through stopcock *B* connected by glass tubing to *S*. The 3 gal. Pyrex Dewar flask (D_1), containing solid carbon dioxide in butyl alcohol is used to cool the drying chamber, and it is arranged so that it can be easily lowered away from the apparatus. A commercially built refrigerator has also been employed in place of solid carbon dioxide for the cooling by Hoerr and Scott (1944). When a pressure of 1 mm. of mercury, or less, is maintained in the space *E*, and paraffin is in tube *C*, the equilibrium temperature over the paraffin is about —66°. As used at present, there is no occasion to employ temperatures higher than —66°, but Packer and Scott point out that a thermocouple sealed in space *E* could be used to operate a thermostat which in turn could control the current in the paraffin heater (*D*) in order to maintain temperatures above —66°. The heater (*D*) is required to melt the paraffin in tube *C* so that the tissues held in the copper gauze basket (C_1) can be embedded *in vacuo*.

The heater (*D*) is constructed by covering a thin-walled copper cylinder with liquid porcelain (Insa-lute), and, after dry, winding No. 18 Nichrome wire (about 70 ohms) over it and applying another layer of liquid porcelain over the wire. A thin-walled sleeve of cop-

per is then fitted over the whole and, after testing the unit, electrical connections to the outside are made through tungsten glass seals. Small glass projections on the outside of the drying tube near the bottom serve to support the heating unit in its proper position.

Two glass boats (*H*) contain the phosphorus pentoxide used as the drying agent. They are placed in tube *G*, which is 4 in. in diameter, through the opening at *J*. The closures at *A* and *J* are *grease joints fitted with springs in the usual manner. All connecting tubes on the low pressure side of the apparatus have a diameter of 1.5 in. The vapor trap (K) has a 1.5 in. inner tube and a 0.5 in. annular space, and the inner tube projects about 5 in. below the* level of the solid carbon dioxide in butyl alcohol used as a refrigerant to surround the trap.

The two-stage diffusion pump (*L*) and the single-stage booster pump (*N*) employ Octoil-S (*Distillation Products Co.*) instead of mercury since the former has a very low vapor pressure (claimed to be $< 10^{-6}$ mm. of mercury at 15°), effects a very high pumping speed, and is much cheaper than mercury. The pump (*L*) has an intake speed of 10 liters per second; it was designed by Professor A. L. Hughes of the Washington University Department of Physics. The boilers of the vapor pumps were protected from drafts, etc. by a covering of several layers of wet asbestos paper and over these a thin aluminum cone (*M*). Turned-under tabs of the cone support a flat circular 300 watt heater (Chromolox). A small electric blower is used to cool the upper chamber of the boiler of pump *L* for maximum efficiency. A single-stage Welch mechanical pump is connected by rubber pressure tubing at *U* to the booster pump (*N*). The phosphorus pentoxide trap (*O*) prevents contamination of the oil when the vacuum is broken by stopcock *P*. The stopcock *Q* has a 1 cm. bore and is used to isolate the high-vacuum section from the mechanical pump while the space *E* is being exhausted. The two-way stopcock *R* connects to the air at *T* and to *E* through *S*.

The type of support employed for the glass apparatus is shown in Figure 2. The main standard is a rectangular aluminum box 4 ft. high bolted to a ¼ in. iron base plate 2 ft. square. The aluminum box is made by welding together at *W* two heavy 8 in. aluminum channels (*V*). The glass is supported by copper rings silver-

soldered to $^5/_{16}$ in. brass rods which are threaded into the aluminum standard. An instrument panel is mounted under *G* to carry the rheostats and meters for controlling the heating current for the diffusion pumps and the paraffin chamber.

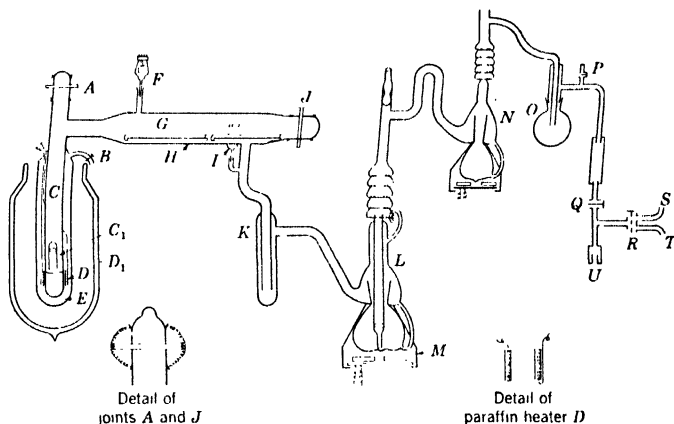



Fig. 1. The Packer-Scott (1942) freezing-drying apparatus.

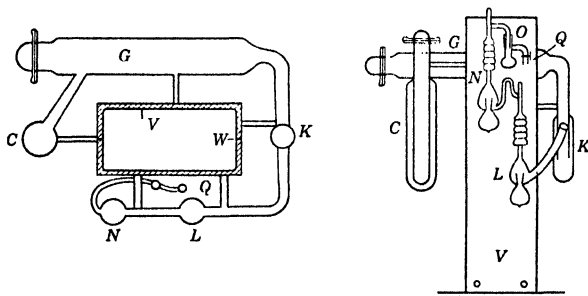



Fig. 2. Arrangement for supporting the Packer-Scott (1942) freezing-drying apparatus.

In order to ascertain something of the state of dehydration of the tissue the ionization pressure gauges *F* and *I* in Figure 1 are fitted into tube *G*. When the rate of evaporation falls to a very

low value, the pressure gradient between F and I disappears. However, since water may diffuse out, it has been found desirable to continue the pumping for a day or more after the pressures at F and I are coincident and remain so.

The ionization gauges used, of the type described by Montgomery and Montgomery (1938), are No. 47 radio tubes of *Radio Corporation of America,* which are sealed to the vacuum system with black vacuum wax, care being taken to avoid knocking off the filament coating during the sealing. A power pack is employed consisting of a half-wave rectifier, filter system, and power transformer. An additional filament transformer is included to supply the filament of the second gauge. In order that the same meters and galvanometer may be used for both gauges a double-throw triple-pole switch is employed. For pressures less than 3×10^{-6} mm. of mercury a student type of wall galvanometer is used to measure positive ion current in the gauges; this current is directly proportional to the pressure. For higher pressures a microammeter with a range of 0–50 may be used. Since the usual calibration constant (1 μamp. for 7×10^{-6} mm. of mercury) is for air, a different constant would apply in the presence of water vapor, hence only relative pressures are obtained.

PROCEDURE

1. Place paraffin in apparatus; melt and degas it *in vacuo* by means of the mechanical pump alone.
2. Let the paraffin solidify and raise the cooling Dewar flask around the drying chamber. When equilibrium is attained the system is ready for the tissue.
3. Either freeze the tissues in liquid air or, preferably, in isopentane at liquid air or liquid nitrogen temperatures. Violently agitate the isopentane to speed the heat extraction.
4. Place frozen tissue in the copper gauze basket and transfer *immediately* to drying chamber of apparatus.
5. Start the mechanical pump at once; then start the diffusion pumps and run for 12 hr. before taking pressure readings. The gauge filaments must be heated and gas allowed to escape for several hours before reliable readings are possible. After the readings of both gauges are equal, continue pumping for some time depending on the size, shape, and character of the tissue. No rule

can be applied here since the time for total dehydration is a function of a number of poorly defined variables.

6. When dehydration is considered complete, lower away the Dewar cooling flask and allow the tissue to come to room temperature.

7. Start the paraffin heater and keep the temperature of the paraffin just above its melting point. The top of the paraffin will melt first; regulate the heating so that a portion of solid paraffin remains at the bottom of the chamber. Conditions of —66° and 4×10^{-7} mm. of mercury are obtained routinely during operation. The tissue will sink into the melted paraffin without causing a single bubble to rise if the tissue is properly dehydrated and the paraffin completely degassed; otherwise bubbling will occur.

8. Break the vacuum after infiltration is complete and before the paraffin has been allowed to solidify; remove and block for cutting.

II. CHEMICAL METHODS

A. REQUIREMENTS

The microscopic technique employing chemical methods depends in almost every case on the direct observation of an insoluble product of a microchemical reaction between the substance or group whose distribution is being investigated and a suitable reagent. Since the whole purpose of these methods is to visualize the presence of a cellular or intercellular constituent *in situ,* it is essential that the tissue be handled in a manner that will not permit the constituent to diffuse or change its anatomical position during the procedure. The minimum requirements of the chemical method then may be listed as:

1. The preparation of microtome sections in which there has been no significant alteration in the position of the constituent being investigated.
2. A reagent which is specific for this tissue constituent.
3. A reaction between the reagent and constituent which is of such a nature, and rapid enough, to obviate diffusion of the constituent or of the reaction product.
4. A reaction product, thus trapped *in situ,* which is capable of being visualized.

The frequency with which these requirements can be met is, unfortunately, still very low. The problem is most difficult in the case of those constituents which are diffusible in solution, *e.g.*, inorganic ions. While it is possible to prepare tissue sections without the use of solutions by means of the freezing-drying technique, the chemical formation of a substance in these sections for purposes of visualization involves the use of a reagent in solution. One might imagine that, if the interaction of the reagent in solution with the ion in the tissue were rapid, the ion would be bound as an insoluble

substance before serious diffusion could occur. Still, in the case of the precipitation of tissue chloride by silver nitrate solution, Scott and Packer (1939) pointed out that differences in ionic mobilities and the effects of ionic charges at cellular interfaces might easily produce precipitations in regions different from those in which the chloride originally existed. On the other hand, Gersh (1941) claimed that the results he obtained for chloride distribution, using silver nitrate as the reagent, were valid as borne out by related data obtained with entirely different biochemical methods. Regardless of the merits in this particular instance, the dangers indicated by Scott and Packer cannot be ignored, and no way has yet been devised to really eliminate them; they constitute a fundamental limitation in the application of the chemical methods of microscopic technique.

In the special case of the localization of enzymes, the sites of activity may be determined in tissue sections by immersing the sections in a buffered substrate medium containing a reagent which will bind one of the products of the enzymatic action *in situ* by precipitation. In addition to the four requirements already listed for determinations of the disposition of tissue constituents, it is also necessary that the following be included for enzyme methods:

5. A reagent which when added to the buffered substrate will react with one of the enzymatic products but not with the substrate or buffer.

6. A reagent which will also have no untoward effect on the enzyme.

7. If the enzymatic product which reacts with the reagent is a substance pre-existing in the tissue, either the sites of enzyme action must be different from those of the pre-existing substance, or the increase in the amount of the visualized compound resulting from the enzyme activity must be demonstrable, or, better yet, the substance must be removed in advance by a method which will not take out the enzyme.

8. A control experiment in which either the substrate is omitted, or a highly effective soluble enzyme inhibitor, such as fluoride, is added (the inhibitor must not react with substrate, buffer, reagent, or products)—the advantage of the inhibitor is that, in some cases, naturally occurring substrate may be present with the enzyme and thus give a false aspect to the nonenzyme control. No such control

experiment is required if all substances pre-existing in the tissue and capable of giving a positive reaction can be removed without seriously reducing the enzyme activity. This has been accomplished in certain tissues for the phosphatase test.

Many, if not most, of the tests described in the following pages leave much to be desired. In some cases they have been developed for particular tissues and cannot be adapted to others without a certain amount of additional research. Most of the tests are clearly not at all good. However, it is the purpose of the writer to present the published methods for the localization of substances, groups, and enzymes, even though they may be, and usually are, poor. In this way the investigator who requires a particular method will have at hand the procedures already developed, and, if they should prove inadequate, at least he will have them as a basis from which to work out improvements.

A word should be added concerning the mounting media employed for tissue sections. The media given in the procedures that follow are those used by the original authors. However, newer media are available and they may be substituted for the balsam or damar that have been employed in the past. A 60% solution of Clarite in xylol appears to be superior to neutral balsam for mounting sections since, according to Lillie (1941), Clarite does not promote the fading of some stains to the degree that balsam does. Stowell and Albers (1943) showed Clarite absorbs less visible light than balsam. Tetrachloroethylene may also be used as a solvent for Clarite. Clarite and Clarite X (also called Nevillite V and Nevillite No. 1, respectively) are superior in all respects to balsam and damar, according to Groat (1939). A solution of 60% of the resin in 40% of toluol by weight is recommended. The resins are clean, cheap, water-white, inert, high-melting, absolutely neutral, and chemically homogeneous. Clarite X undergoes a slight yellowing with age and has a refractive index of 1.567 while Clarite is very color stable and has a refractive index of 1.544.

The limited availability of certain reagents or enzyme substrates may make it imperative to employ a considerably smaller volume than is normally used in staining dishes and Coplin jars. The hanging-drop technique (Glick and Fischer, 1945a) may be adopted in these cases. The section on the slide is surrounded by a circle of vaseline or stopcock grease, a drop of the reagent or substrate

solution is placed on the section, and to avoid evaporating when prolonged contact between the liquid and the section is necessary, a hanging-drop slide is placed over the section so that the drop is enclosed by, but does not touch, the walls of the depression. The two slides are bound together with a rubber band and carefully inverted so that the section is covered by the hanging-drop.

B. INORGANIC ELEMENTS AND RADICALS

POTASSIUM

Macallum's original method for the histochemical detection of potassium based on the precipitation of sodium potassium cobaltinitrite has been subject to modifications over the past thirty years. The relatively recent modification of Gersh (1938) will be included in the present work as well as the method of Carere-Comes (1938), which depends on the development of an orange color with Siena orange.

Gersh Modification of Macallum Method for Potassium

SPECIAL REAGENTS

Anhydrous Petroleum Ether freshly distilled over sodium (20–40° b.p.).

Dried Paraffin (Grübler, 50–52° m.p.). Just before use heat at 100° or more *in vacuo* for about 15 min. or until bubbling stops.

12% Sodium Cobaltinitrite Solution. Dissolve 150 g. sodium nitrite in 150 ml. hot water, cool to 40° (some crystals appear), add 50 g. cobalt nitrate crystals, while stirring rapidly add 50 ml. 50% acetic acid in small portions, stopper, and shake well. Pass a rapid stream of air through the solution and let stand overnight. Siphon off clear liquid and filter. Add 200 ml. alcohol in small portions to the filtrate with stirring. After a few hr. filter off precipitate by suction. Wash precipitate four times with 25 ml. portions of alcohol followed by two times with ether. Recrystallize by dissolving each 10 g. solid in 15 ml. water and precipitate with 35 ml. alcohol. Make up the 12% aqueous solution fresh before use.

PROCEDURE

1. Subject tissue to freezing-drying (see page 3).
2. Transfer to paraffin, infiltrate *in vacuo* for 10–15 min. at not more than 60°, and embed. Care should be taken to prevent condensation of moisture on the dried tissue during the transfer from the vacuum vessel to the paraffin.
3. Cut 15 μ sections using no water or ice, mount near edges of large chemically clean cover slips, press down with finger, melt paraffin with a tiny flame, and press down again.
4. Remove paraffin by immersing the cover slips with the sections in dry petroleum ether in a watch glass heated on a warm bar. (Keep watch glass covered by another at all times except during actual use. Replace the petroleum ether often.)
5. Remove from petroleum ether and burn off excess quickly. Allow to cool.

From this point on, the test is carried out entirely in a cold room the temperature of which should be 0° ± 1° during the manipulations. All instruments and reagents are kept in this room. The crystals of sodium potassium cobaltinitrite are relatively soluble at room temperature, hence the precautions to maintain cold.

6. When cover slips with sections are cold, cover each section with a drop of the sodium cobaltinitrite solution.
7. Drain off the solution and replace with glycerol.
8. Mount on clean slide with section between slide and cover slip.
9. Examine under microscope with oil immersion lens. (Light mineral oil is substituted for cedar oil since the latter is too viscous at 0°.)

Result. Short yellow rods with rounded ends in a diffuse pale yellow background are the crystals of sodium potassium cobaltinitrite.

Carere-Comes Siena Orange Method for Potassium

SPECIAL REAGENTS

Siena Orange Solution. Aqueous sodium *p*-dipicrylamine (prepared ready for use by *K. Hollborn & Sons*).

10% Hydrochloric acid.

PROCEDURE

1. Fix tissue in neutral formalin and prepare paraffin sections as usual.
2. Immerse deparaffinized sections in Siena orange soln. for 2 min.
3. Transfer to 10% hydrochloric acid for 3 min.
4. Wash twice in distilled water for 10 min., blot with filter paper, and dry at 37°.
5. Mount in thickened cedar oil.

Result. Potassium is demonstrated by an orange color on a pale yellow or colorless background. The author of this method has failed to consider the effects of potassium diffusion when aqueous solutions are employed for fixation, etc. Modification of the procedure to obviate this difficulty would be essential.

CALCIUM

A critical survey of histochemical tests for calcium was presented by Cameron (1930), who concluded that none of the tests can be considered wholly specific. In all cases calcium must be converted to an insoluble salt, if it is not already present as such, and the insoluble compound is identified directly or it is made more easily detectable by staining or conversion to a colored compound. For visualization of calcium in the form of phosphate or carbonate see page 78. In addition to these tests the Crétin (1924) gallic acid color test has been extensively used, as has the formation of a red precipitate by reaction of calcium salts with sodium alizarin sulfonate (Pollack, 1928). For plant materials it is often sufficient to produce and identify crystals of the oxalate, carbonate, or sulfate (Lee's *Vade Mecum,* pages 293 and 668). The old test of von Kóssa (1901), depending on the reduction of silver salts under bright light, has been championed by Gomori (1945a). While this method will demonstrate inorganic deposits in general, it can be considered specific for calcium in bone or cartilage because the calcium salts are the only ones present in significant amounts. An adaptation of the von Kóssa test to bone has been described by McLean and Bloom (1940) and Bloom and Bloom (1940).

Calcium Sulfate Test for Calcium in Plant Tissue

SPECIAL REAGENTS

3% Sulfuric Acid.
40% Alcohol.

PROCEDURE

1. Fix tissue in acid-free alcohol or acid-free formalin.
2. Cut sections and bring them down to 40% alcohol.
3. Add 3% sulfuric acid to sections under cover slip.
4. Examine for colorless monoclinic needles of calcium sulfate.

Crétin Color Test for Calcium and Other Minerals

SPECIAL REAGENTS

Gallic Acid Reagent. Grind 0.1 g. trioxymethylene (metaformaldehyde) and 0.2 g. gallic acid in a mortar. Dissolve 0.25 g. of the mixture in 5 ml. boiling distilled water and add 0.5 ml. ammonium hydroxide (18° Baumé, or 14% ammonia); stir until the solution becomes straw colored. When this reagent turns brown or rose, which it will in a short time, it can no longer be used.

PROCEDURE

1. Prepare paraffin sections as usual. Remove the paraffin with xylol and the xylol with chloroform.

2. After excess chloroform has been removed, add gallic acid reagent. In 10–15 sec., drain off excess reagent and expose slide to air.

3. Examine when color appears. An eosin counterstain may be applied (Lison, 1936, page 76).

Result. Calcium gives a blue, barium a bright green, strontium a water blue-green, cadmium a bluish-green, magnesium a yellowish-rose, iron a deep violet-brown, zinc and lead a dull yellow, and silicon a pure yellow color.

Von Kóssa Silver Test for Calcium

SPECIAL REAGENTS

5% Silver Nitrate.

PROCEDURE

1. Transfer frozen or paraffin sections which have been washed with distilled water to the silver nitrate soln. in the dark for up to 1 hr.; wash with distilled water in the dark, and expose to bright light for 30 min. or longer.
2. Wash well in distilled water, dehydrate, clear, and mount.

Result. Calcium salts are rendered black.

MAGNESIUM

A method for the demonstration of magnesium in plant cells was developed by Broda (1939). The principle of this method could be adapted to studies on animal tissue. Most of the tests used for calcium also give positive results for magnesium.

Broda Method for Magnesium

SPECIAL REAGENTS

Quinalizarin Reagent. Triturate 100 mg. quinalizarin and 500 mg. sodium acetate crystals, and dissolve 500 mg. of the mixture in 100 ml. of 5% sodium hydroxide.

0.2% Titian Yellow.

10% Sodium Hydroxide.

0.1% Azo Blue.

PROCEDURE

1. Prepare paraffin sections as usual.
2. Add 1–2 drops of quinalizarin reagent to a section on the slide, followed by 1–2 drops of 10% sodium hydroxide.
3. To a different section add 1–2 drops of the Titian yellow solution followed by 1–2 drops of 10% sodium hydroxide.
4. To another section add a drop or two of the azo blue dye.

Result. In the presence of magnesium the quinalizarin reagent develops a blue color over several hours, the Titian yellow a brick-red, and the azo dye a violet stain.

ZINC

Very little has been done in regard to the histological localization of zinc and the procedure of Mendel and Bradley (1905) is still

the sole method that has been developed. Zinc is precipitated by nitroprusside and the precipitate is brought out as a deep purple by treatment with sulfide.

Mendel and Bradley Method for Zinc

SPECIAL REAGENTS

10% Sodium Nitroprusside.

Potassium Sulfide Solution. (Concentration not stated, but 1–5% should suffice.)

PROCEDURE

1. Prepare paraffin sections. (Mode of fixing tissue not given, but, as in all other cases, the freezing-drying treatment, see page 3, would be preferable.)
2. Treat sections with the nitroprusside soln. for 15 min. at 50°.
3. Cool, and wash in a stream of water for about 15 min.
4. Introduce under cover glass placed on section 1 drop of the sulfide soln.

Result. Zinc elicits an intense purple color.

IRON

The classical histochemical tests for iron are the Prussian and Turnbull's blue reactions and the hematoxylin method of Macallum, the latter being the least specific (Lee's *Vade Mecum*, pages 289–292). The Prussian blue test will detect ferric, and Turnbull's blue ferrous, iron. More recently other methods have been proposed for which certain advantages have been claimed. The dinitrosoresorcinol test of Humphrey (1935) brings out iron as a rich green of pristine brilliance and the color is much more permanent than that of Prussian or Turnbull's blue, which fades after a year or two. Thomas and Lavollay (1935) employed the 8-hydroxyquinoline reaction to visualize iron in greenish-black; other metals appearing in various shades of green and yellow. The strong flourescences of metallic 8-hydroxyquinolinates may also be used for identifications (see page 108).

Iron, like many other metals, occurs in tissues both in the inorganic or free form, and in the organic or bound form. Before bound iron can be visualized it must be converted to the free form.

Macallum's (1908) technique is still employed for this conversion and it consists of a treatment of deparaffinized sections with a solution of either nitric or sulfuric acid in alcohol. The iron liberated is chiefly in the ferric form. In all tests special care must be taken to protect tissues and fluids from dust. Iron will also appear in the tests for lead and copper (see page 22).

Precautions to prevent diffusion of the iron in aqueous solutions have not been sufficiently exercised in the following procedures. The investigator should modify them accordingly.

Prussian Blue Test for Ferric Iron and Turnbull's Blue for Ferrous Iron

SPECIAL REAGENTS

Prussian Blue Reagent. 2% potassium ferrocyanide (use fresh soln.).

Turnbull's Blue Reagent. 2% potassium ferricyanide (use fresh soln.).

Acid Alcohol. 1% hydrochloric acid in 70% alcohol.

Organic Iron Reagent. Equal vol. of 1.5% potassium ferrocyanide and 0.5% hydrochloric acid (use fresh soln.).

Organic Iron Conversion Reagent. 3% nitric acid, or 4% sulfuric acid, in 95% alcohol. (The sulfuric reagent acts more slowly.)

PROCEDURE FOR INORGANIC IRON

1. Fix in 95% alcohol for 24–48 hr.

2. Prepare paraffin sections as usual (care must be taken to minimize contact with iron—the microtome knife must be free of rust and not freshly honed and glass needles should be substituted for the steel ones).

3. After removing paraffin and passing down to distilled water, place sections in either the Prussian or Turnbull's blue reagent for 3–15 min. (If both ferric and ferrous iron are to be visualized, use a mixture of equal vol. of the two reagents.)

4. Wash in water containing eosin or safranin to counterstain.

5. Dehydrate, clear, and mount in benzol balsam.

PROCEDURE FOR ORGANIC IRON

1–2. Same as inorganic iron.

3. Liberate iron from the bound forms by treating deparaffinized

sections, brought down to water, with the conversion reagent for 24–36 hr. at 35°.

4. Wash in 90% alcohol followed by distilled water.

5. Place in the organic iron reagent for not over 5 min.

6–7. Same as 4–5 for inorganic iron.

Result. The iron appears blue.

Humphrey Dinitrosoresorcinol Test for Iron

SPECIAL REAGENTS

30% Ammonium Sulfide.

Saturated Aqueous Dinitrosoresorcinol or a 3% soln. in 50% alcohol. (A few days aging improves the reagent; it is stable.)

PROCEDURE

1. Prepare formalin-fixed paraffin sections.

2. Remove paraffin; bring down to water, and place in the ammonium sulfide solution for 1 min.

3. Rinse in distilled water and place in the dinitrosoresorcinol reagent for 6–20 hr. depending on the depth of the brown background desired.

4. Wash in water or dilute alcohol depending on whether an aqueous or alcoholic reagent was used.

5. Pass through alcohols, carboxylol and xylol, and mount in balsam.

Result. The iron appears bright dark green against a reddish or rich brown background. For organic iron introduce steps 3–4 in Prussian blue procedure (see page 20) between steps 1 and 2 above.

Thomas and Lavollay Hydroxyquinoline Test for Iron

SPECIAL REAGENTS

Hydroxyquinoline Reagent. Dissolve 2.5 g. 8-hydroxyquinoline in 4 ml. glacial acetic acid with the aid of gentle warming. Quickly add distilled water to bring volume to 100 ml. and filter the soln.

25% Ammonium Hydroxide.

PROCEDURE

1. Fix tissue in alcohol, neutral formalin, or trichloroacetic acid soln.

2. Prepare frozen or paraffin sections as usual.

3. To the sections washed with neutral distilled water add a few drops of the hydroxyquinoline reagent, and after 5–15 min. drain off the liquid.

4. Add 1 drop of 25% ammonium hydroxide to form precipitate.

5. Wash in neutral distilled water. No large crystals should remain.

6. A lithium carmine nuclear stain may be applied.

7. Dehydrate with terpinol and mount in petrolatum, or examine directly in neutral water.

Result. Iron appears as a greenish-black, calcium as a pale yellow, magnesium as a straw-yellow, aluminum as a yellowish-green, zinc and manganese as a yellow, and copper as a greenish-yellow precipitate.

NICKEL

A method has been devised by Crétin and Pouyanne (1933) for the histological demonstration of nickel in bone material by precipitation of nickel ammonium phosphate.

Crétin and Pouyanne Method for Nickel

SPECIAL REAGENTS

Fixative. Add 30 ml. formalin and 5 drops ammonium hydrosulfide to 100 ml. physiological saline soln.

10% Ammonium Phosphate.

PROCEDURE

1. Fix tissue in the special fixative soln.
2. Transfer to the ammonium phosphate soln. in order to precipitate the insoluble nickel ammonium phosphate.
3. Decalcify and section.
4. Stain the nickel compound with alcoholic hematoxylin.
5. Wash, dehydrate, clear, and mount.

Result. Nickel will appear as a lilac deposit, or blue if present in abundance.

LEAD AND COPPER

For many years the chromate method has been used for the microchemical detection of lead in tissues (Frankenberger, 1921; Cretin,

1929). This procedure depends on the formation of a yellow precipitate of lead chromate when lead-bearing tissue is fixed in Regaud fluid (20 ml. of 3% potassium dichromate plus 5 ml. formalin). Lison (1936, page 101) has discussed this method as well as the test based on precipitation of the sulfide, and rather favors the former. Okamoto and Utamura (1938) employed *p*-dimethylaminobenzylidene rhodanine to produce a reddish-violet precipitate with copper in tissues, a reaction given by gold, silver, and other metals (see pages 26, 28, and 29).

Mallory and Parker (1939) described a method using hematoxylin and another employing methylene blue which would visualize both lead and copper. The methylene blue technique was particularly recommended for photomicrography of lead because of the intense blue color developed.

In a study of the histological distribution of copper in the blowfly, Waterhouse (1945) found that the only reagent which could be used, of those tested, was sodium diethyl dithiocarbamate, which formed a yellow product with copper. Waterhouse's technique was to drop a 0.1% aqueous solution on the fresh tissue followed by a drop of concentrated hydrochloric acid. The acid allowed greater penetration of the reagent into the cells. Iron can interfere with this test by the formation of a brown carbamate; however, the reagent can detect 1 part of copper in 100 million and its sensitivity to iron does not approach this.

Mallory and Parker Hematoxylin Method for Lead and Copper

SPECIAL REAGENTS

Hematoxylin Reagent. Dissolve 5–10 mg. hematoxylin in a few drops of 95% or absolute alcohol and add 10 ml. freshly filtered 2% dibasic potassium phosphate.

PROCEDURE

1. Fix tissue in 95% or absolute alcohol (formalin may be used for copper).
2. Prepare celloidin sections as usual.
3. Stain sections for 2–3 hr. at 54°.
4. Wash in several changes of tap water 10 to 60 min.

5. Dehydrate in 95% alcohol, clear in terpinol, and mount in terpinol balsam.

Result. Lead appears as light to dark grayish-blue and nuclei as deep blue. Copper or hemofuscin pigment is brought out as an intense blue. Inorganic iron or the pigment, hemosiderin, appears black provided alcohol was used as the fixative and light to dark brown if formalin was employed.

Mallory and Parker Methylene Blue Method for Lead and Copper

SPECIAL REAGENTS

Methylene Blue Reagent. 0.1% of the dye in 20% alcohol.

PROCEDURE

1. Fix tissue in Zenker fluid.
2. Prepare paraffin sections as usual, and apply a contrast stain of phloxine if desired.
3. Treat sections for 10–20 min. with the methylene blue reagent and decolorize in 95% alcohol for about the same time.
4. Dehydrate, clear, and mount as usual.

Result. Lead is colored intense blue. Copper or hemofuscin appears pale blue while iron pigment is not colored and hence appears yellow to light brown. When pigment has both copper and iron it develops a green color.

MERCURY

Three methods for the visualization of mercury in tissue sections are given in Lison (1936, page 102). The mercury can be transformed into the black sulfide, reduced by stannous chloride to give the black free metal, or a violet precipitate can be formed with diphenylcarbazide. In addition to these, Okamoto's method for silver (page 26) using *p*-dimethylaminobenzylidene rhodanine can be employed to give a reddish-violet precipitate with mercury.

After trials of the sulfide, diphenylcarbazide, and reduction methods, Hand *et al.* (1943) favor the latter. They detected mercurous mercury by reducing it to the metal by means of thioglycollic acid, and the mercuric form was visualized by reducing with stannous chloride.

Method of Hand *et al.* for Mercurous and Mercuric Mercury

SPECIAL REAGENTS

Mercurous Reagent. Combine 1 ml. thioglycollic acid with 9 ml. glycerol.

Mercuric Reagent. Combine 5 g. stannous chloride, 5 g. tartaric acid, and 100 ml. glycerol, and heat until clear. Stabilize by adding a few grams of metallic tin to the final soln., which should be stored in a stoppered bottle.

Iodine Reagent. Dissolve 50 g. potassium iodide in 50 ml. distilled water; add 70 g. iodine and when it has dissolved, add 95% alcohol to make 1 liter.

1% Chloroauric Acid. Store in a dark bottle.

Control Reagent. Add 5 g. tartaric acid to 100 ml. glycerol. Let stand overnight to dissolve.

PROCEDURE

1. Prepare fresh frozen sections of tissue 15 μ thick.
2. Place sections on slides and allow to dry.
3. Cover each section with a drop of one of the reagents, depending on the test to be applied, fit on a cover slip, and blot away excess reagent.
4. Seal edges of cover slip with commercial gold size (adhesive used to hold gold foil on glass).
5. After 10 min. examine under a microscope, comparing sections with control reagent to those with other reagents. The sections treated with the mercuric and control reagents remain unchanged for at least 2 weeks.

Result. The metallic mercury formed in the tissue appears as minute black spheres which may be dissolved by tincture of iodine, or made to lose their glossy surface by forming gold amalgam on treatment with chloroauric acid. In the test for mercurous mercury characteristic yellowish crystals appear after about 5 min., in addition to the mercury globules, when mercuric mercury is also present.

SILVER

Particles of reduced silver in tissues may be made more intensely black by treatment with dilute ammonium sulfide solution.

Okamoto, Utamura, and Akagi (1939) employed the *p*-dimethyl-

aminobenzylidene rhodanine reagent for the precipitation and visualization of silver in tissue sections. The fact that not only silver, but also copper, gold, mercury, platinum, and palladium are likewise precipitated by this reagent offers little ground for concern since these elements are not apt to exist in significant amounts in tissues unless introduced experimentally or perhaps by accidental poisoning. In these cases only one of the elements at a time is likely to be present. However, certain differentiations can be made, if it is assumed that more than one is present, on the basis of the varying solubility behavior of the precipitated compounds. Thus divalent copper reacts with the reagent only in neutral solution, whereas monovalent copper and the other metals will react in either neutral or acid solution. Furthermore the mercury precipitate is soluble in dilute hydrochloric acid, the silver compound in potassium bromide solution, and gold compound in potassium nitrite solution. Neutral stannous chloride solution reduces the palladium precipitate to the free metal which can then be converted to the chloride by means of chlorine gas; this cannot be done with the platinum compound. Based on these facts, possible separations have been suggested by Okamoto *et al.*

Okamoto *et al.* Procedure for Silver

SPECIAL REAGENTS

Precipitation Reagent I. Add 3–5 ml. of saturated soln. of *p*-dimethylaminobenzylidene rhodanine in absolute alcohol to 1–3 ml. 1 *N* nitric acid and 100 ml. distilled water.

Precipitation Reagent II. Combine 10–20 ml. of the saturated alcoholic soln. of the rhodanine compound with 1–3 ml. 1 *N* nitric acid, 5–10 ml. 3% hydrogen peroxide, and 100 ml. distilled water.

Precipitation Reagent III. Add 2–5 ml. of the soln. of the rhodanine derivative to 2 ml. 0.1 *N* hydrochloric acid, 3–5 ml. 1 *N* nitric acid, and 100 ml. distilled water.

PROCEDURE

1. After fixing the tissue in absolute alcohol or neutral formalin, prepare either celloidin, paraffin, or frozen sections.
2. Place the sections in precipitation reagent I for 24 hr. at 36°, keeping the vessel closed.

3. Rinse sections in distilled water and counterstain with hematoxylin.
4. Dehydrate, clear, and mount in balsam.

Result. Silver in the tissue is colored reddish-violet. The other metals will produce shades of the same color.

Monovalent copper may be eliminated from visualization by substituting precipitation reagent II for I, and mercury may be similarly eliminated by employing reagent III. The silver precipitate could be removed from the others by treating the colored sections with 1% potassium bromide.

GOLD

Several methods have been used for the localization of gold in tissues and Lison (1936, page 100) has discussed those of Christeller, of Borchardt, and of Okkels. The first depends on treatment with stannous chloride; the second employs silver nitrate followed by nitric acid, and the last merely involves exposure to ultraviolet light to obtain blackening of the gold granules. The more recent methods of Roberts (1935), Okamoto, Akagi, and Mikami (1939), and Elftman and Elftman (1945) follow. The last-named method is probably the best since it avoids the use of ions that might give rise to artifacts, and effects the bleaching of interfering pigments.

Roberts Method for Gold

SPECIAL REAGENTS

Silver Nitrate Reagent. Just before using dissolve 2 g. pure silver nitrate in 100 ml. 10% gum arabic soln. in the dark.

Hydroquinone Reagent. The day before using dissolve 1 g. pure hydroquinone in 100 ml. 10% gum arabic soln.

5% Citric Acid.

5% Sodium Hyposulfite.

PROCEDURE

1. Fix tissue in Bouin fluid or 20% neutral formalin and wash well with water.
2. Prepare paraffin or frozen sections.
3. Place sections for 5–10 min. in a fresh mixture of 2 ml. silver nitrate reagent, 2 ml. hydroquinone reagent, and 1–3 drops of 5%

citric acid. Shake the mixture for 30 sec. immediately after preparing it.

4. Transfer sections rapidly to a 5% sodium hyposulfite soln. and after a few min. wash thoroughly in water.

5. Dehydrate, clear, and mount.

Result. Granules of gold appear black due to a surface deposit of silver.

Elftman and Elftman Method for Gold

SPECIAL REAGENTS

3% Hydrogen Peroxide.

PROCEDURE

1. Fix tissue in neutral formalin, prepare paraffin sections, mount with the aid of egg albumin, and then expose to formaldehyde vapor for 1 hr. to increase the affixation.
2. After removing the paraffin and running down to water, place in 3% hydrogen peroxide at 37° for at least 24 hr.; in most cases 3 days or longer gives the best results.
3. Do not stain the sections since staining may mask the gold deposit. If staining is required, however, the interference is only slight with light green SF (yellowish) or hematoxylin, and eosin can be used when the gold is present as a sufficiently dense deposit.
4. Wash the sections in distilled water and dehydrate in the usual manner.
5. Mount the sections in damar.

Result. Gold is made apparent by its presence in colloidal form. The color of the deposit depends on the particle size, and accordingly shades from rose to purple to blue and black are obtained. Usually rose predominates.

Okamoto *et al.* Method for Gold

The procedure is the same as that in the Okamoto *et al.* method for silver using precipitation reagent II (page 26). The gold appears as a reddish-violet or brownish-red precipitate. The removal

of the colored silver precipitate from the sections, if silver was present, can be carried out by treating with a saturated soln. of potassium bromide for 1 hr. or more. If the sections are then washed in distilled water and placed in 1% potassium nitrite for 24 hr. or longer at 36° (or heated in the nitrite soln. for 1 min.) the gold precipitate will dissolve leaving those of platinum or palladium, should either of these be present.

PLATINUM

The Okamoto *et al.* method for silver may be employed unchanged for the detection of platinum in tissues (see page 26).

PALLADIUM

The method of Okamoto, Mikami, and Nishida (1939) for the visualization of palladium in sections of tissue follows the Okamoto *et al.* silver method (p. 26) with 1 difference. Between steps 1 and 2 in the procedure, the following is introduced: treat the dry sections with chlorine gas until the black palladium granules are made colorless.

URANIUM

Two chemical tests have been presented for the localization of uranium in tissue sections. Both are founded on the precipitation of dark brown uranium ferrocyanide. Schneider (1903) was the first to use this technique on the tissues of animals that had been injected with uranium salts. Gérard and Cordier (1932) followed the Prussian blue method for iron and reported good results. The latter employed Bouin-Hollande or Carnoy fixatives and their coloring reagent was 2% potassium ferrocyanide containing 2% hydrochloride acid. For details of the test, see the Prussian blue procedure for iron, page 20.

The fluorescent properties of uranium salts subjected to ultraviolet radiation can be utilized for the detection of these salts in incinerated sections of tissue as indicated by Policard and Okkels (1930); see page 145.

ARSENIC

Castel (1934–1935a, 1936) developed two methods for the histological localization of arsenic. In one the tissue is fixed in an absolute alcohol-chloroform-hydrochloric acid mixture saturated with hydrogen sulfide, and the appearance of yellow granules was believed by Castel to be due to the formation of arsenous sulfide. A reinvestigation of this technique by Tannenholz and Muir (1933) led them to conclude that the yellow granules formed were not related to the presence of arsenic but were more likely composed of a sulfur-protein complex.

The other method of Castel was based on the precipitation of either cupric hydrogen arsenite (Scheele's green) or the cupric acetate-cupric arsenite double salt (Schweinfürter green), and this procedure appears to be a reliable one.

Castel Cupric Salt Method for Arsenic

SPECIAL REAGENTS

Formalin-Copper Salt Reagent. Add 2.5 g. cupric sulfate or neutral cupric acetate to 100 ml. metal-free 10% formalin (hydrogen sulfide is used to test for traces of metals in the formalin).

PROCEDURE

1. Place small pieces of tissue in the formalin-copper salt reagent for 5 days.
2. Wash tissue in running water for 1 day.
3. Prepare paraffin sections as usual and examine after removal of the paraffin.

Result. Green granules are indicative of arsenic.

BISMUTH

The histochemical detection of bismuth is founded on the reaction of Léger (1888), which is the precipitation of the double iodide of bismuth and an alkaloid. Komaya (1925) and Christeller (1926) employed the quinine salt for their tissue studies, and later Castel (1936) suggested the use of the brucine salt to avoid the interference of iron which plagues the quinine method. He also modified the earlier procedures by substituting sulfuric for nitric acid in the reagents, Castel (1934–1935b), a change which enables the visual-

ization of the bismuth as a more reddish, rather than a yellowish-orange deposit. More recently Wachstein and Zak (1946) employed a modified Castel method in which the black sulfide, the form in which bismuth appears in tissues, is converted to the white sulfate by treatment with hydrogen peroxide and the sulfate is then transformed to the brucine iodide salt.

In the procedure of Wachstein and Zak (1946), iron, which may be present as the black sulfide, is oxidized to golden brown hemosiderin, which does not react with Castel reagent, but which does give the typical iron reactions. Wachstein and Zak pointed out that lead sulfide would deposit in tissues in the same fashion as bismuth but differentiation may be made by the fact that, after the lead sulfide is converted to the sulfate by the peroxide, it will yield the slightly yellowish lead iodide on treatment with Castel reagent in contrast to the brilliant orange-red bismuth product. Similarly silver and mercury will give yellow, and copper brown, iodides that can be differentiated from the color of the bismuth precipitate. Melanin in tissue is not bleached by the short treatment with peroxide and does not react with Castel reagent. Wachstein and Zak emphasized that melanin never impregnates capillary walls while bismuth does.

Wachstein and Zak Method for Bismuth

SPECIAL REAGENTS

Modified Castel Reagent. Dissolve 0.25 g. brucine sulfate in 100 ml. distilled water containing 2–3 drops conc. sulfuric acid. After the brucine salt has dissolved add 2 g. potassium iodide. Store in a brown bottle and filter before use.

Diluted Castel's Reagent. Add 3 vol. distilled water to 1 vol. reagent.

30% Hydrogen Peroxide. (Superoxol, *Merck*). Store in a refrigerator.

Levulose Solution. Dissolve 30 g. levulose in 20 ml. water by warming at 37° for 24 hr. and add a drop of the diluted Castel reagent.

Counterstain Solution. Add 1 ml. 1% aqueous light green SF (*Hartman-Leddon*) to 100 ml. undiluted Castel reagent. Filter before use.

PROCEDURE

1. Prepare either frozen or paraffin sections of formalin-fixed tissue.

2. Treat the tissue on the slide for a few sec. with several drops of 30% hydrogen peroxide to remove the black sulfide color.

3. Wash well in tap water and place in the Castel reagent for 1 hr.

4. Transfer to the diluted Castel reagent and shake gently to remove precipitates.

5. Remove most of the liquid from the slide by careful blotting and mount in the levulose soln.

Result. Bismuth is indicated by the orange-red deposit. The color may darken on standing. (If a counterstain is desired, stain for 4 min. with the counterstain soln.)

Castel Method for Bismuth

SPECIAL REAGENTS

Bismuth Reagent. With the aid of warming, dissolve 1 g. brucine in 100 ml. distilled water containing 3–4 drops of sulfuric acid and add 2 g. potassium iodide. As an alternate reagent dissolve 1 g. brucine and 2 g. potassium iodide in 100 ml. of a mixture of equal vol. alcohol and chloroform.

PROCEDURE

1. Fix the tissue in 10% formalin and prepare paraffin sections.

2. Treat deparaffinized sections for 15 min. with the bismuth reagent and wash well in distilled water.

3. Mount in syrup of Apáthy (heat equal parts of paraffin, m.p. 60°, and Canada balsam).

Result. Red granules are indicative of bismuth.

CHLORIDE AND PHOSPHATE-CARBONATE

Earlier methods for chloride, including Macallum's (1908) silver test, were subject to the difficulty that, in the course of the manipulations, a shift in the topographical distribution of chloride occurred. Distortion due to this cause can be minimized by applying the freezing-drying process to the tissue before further treatment. Gersh (1938) makes use of this fact in his procedure, which enables a differentiation between the chloride in the tissue and the phosphate and carbonate present. Phosphate and carbonate are visualized together in this method. Two reagents are used, one permits visualization of chloride specifically by effecting the maximum precipita-

tion of chloride in the presence of phosphate and carbonate by means of the phosphoric acid it contains. The acid holds the phosphate in solution and decomposes the carbonate. The other reagent, without phosphoric acid, precipitates chloride, phosphate, and carbonate. By comparing sections separately treated with each reagent, chloride can be differentiated from phosphate and carbonate.

Gersh Method for Chloride and Phosphate-Carbonate

SPECIAL REAGENTS

Anhydrous Petroleum Ether freshly distilled over sodium (b.p. 20–40°).

Dried Paraffin (Grübler, m.p. 50–52°). Just before use heat at 100° or more *in vacuo* for about 15 min. or until bubbling stops.

Silver Nitrate Reagent 1. To 60% silver nitrate solution add enough conc. phosphoric acid to prevent precipitation of high concentrations of phosphate, then saturate with silver chloride. Filter and add 2–3 drops distilled water to each 10 ml. before using.

Silver Nitrate Reagent 2. Saturate 60% silver nitrate solution with silver phosphate and silver chloride. Filter and add water before using as for reagent 1. Store both reagents in glass-stoppered brown bottles in the dark.

PROCEDURE

1–5. These steps are identical with those in Gersh method for potassium (see page 14).

6. Cover sections on one cover slip with reagent 1 and those on another with reagent 2.

7. Drain off liquid from both cover slips and replace with a drop of pure glycerol in each case.

8. Mount on clean slides with glycerol-covered sections down.

9. Expose both slides simultaneously to carbon arc radiation at such distance as to avoid warming .

10. Examine microscopically at once by direct or dark-field illumination. These preparations last only a short time. The highest power to be used with the dark-field condenser is a 4 mm. high-dry or 2 mm. oil immersion objective with a numerical aperture of 0.95.

Result. The reduced silver appears yellow to brown with or without black or brown particles when viewed with direct illumination.

With the dark field the silver granules appear orange or rust colored. Reagent 1 gives a test for chloride only. Reagent 2 gives a test for chloride plus phosphate and carbonate.

IODIDE

A critical study of histochemical methods for the localization of iodides in tissues was presented by Gersh and Stieglitz (1933). After a careful examination, these authors conclude that none of the proposed methods is satisfactory. The difficulty is that any precipitating agent that might be used to fix iodide will also precipitate the proteins and hence prevent its proper penetration into the tissue.

PHOSPHATE

Microscopic techniques for the detection of phosphorus in tissues are usually based on reactions designed to visualize the phosphate ion. Hence phosphate in organic combination must be liberated before it can be detected. Lison (1936, pages 113–120) critically reviewed the various methods and considered Angeli's procedure to be highly specific; this is a molybdate method using stannous chloride as the reducing agent. Serra and Queiroz Lopes (1945) employed a molybdate reaction with benzidine which they report to give a more intense color than that developed by stannous chloride, and they also use a more dilute acid medium, which is less damaging to the tissue.

Johansen (1940, page 198) stated that phosphate may be identified in plant tissues by treating a section with a drop of solution prepared by adding 25 ml. saturated magnesium sulfate and 2 ml. saturated ammonium chloride to 15 ml. water. Crystals of magnesium ammonium phosphate should form in the presence of phosphate. This procedure is doubtlessly less advantageous than those previously mentioned.

The method for visualization of enzymatically liberated phosphate (page 78) may also be applied in certain instances.

Serra and Queiroz Lopes Modification of the Molybdate Method for Phosphate

SPECIAL REAGENTS

Acetic Alcohol-Formalin Fixative. Add a few drops of glacial

acetic acid to 10 ml. of a mixture of 2 vol. 96% alcohol and 1 vol. formalin.

Molybdate Solution. Dissolve 0.5 g. ammonium molybdate in 20 ml. distilled water, add 10 ml. conc. (30%) hydrochloric acid, and dilute to 50 ml. with distilled water.

Acetic Benzidine Solution. Dissolve 25 mg. benzidine in 5 ml. glacial acetic acid and dilute to 50 ml. with distilled water.

Saturated Sodium Acetate Solution.

PROCEDURE

1. Fix the tissue in the acetic alcohol-formalin mixture and wash well in water.

2. In order to hydrolyze organic phosphates and precipitate the free phosphate, treat small pieces of the tissue or frozen sections with the molybdate reagent at 10–12° for 2–3 weeks, followed by 2–3 days at 20–25°. The temperature is kept low to prevent alteration of the tissue and the rather long time is required to effect hydrolysis with the relatively weak acid concentration of the reagent.

3. Cover the tissue with a drop of acetic benzidine soln. for 3 min. and then add 2 drops of the sodium acetate soln.

4. Mount in glycerol which has been stored with crystals of sodium acetate in the bottle.

Result. An intense blue coloration characterizes phosphate.

NOTE: Serra and Queiroz Lopez employ a digestion with nuclease to liberate phosphate from nucleic acid. The visualization of this phosphate then serves to indicate the nucleic acid.

NITRATE

Crämer (1940) developed a method for the histological demonstration of nitrate which is based on the doubly refractive property in polarized light of the insoluble salt formed by the interaction of nitrate with Nitron (4,5-dihydro-1,4-diphenyl-3,5-phenylimino-1,2,4-triazole). Busch (1905) originally employed this reaction for the gravimetric determination of nitric acid.

Crämer Method for Nitrate

SPECIAL REAGENTS

Nitron Reagent. 10% Nitron in 5% acetic acid.

PROCEDURE

1. Prepare frozen sections of fresh tissue.
2. Place 1–2 drops of hot Nitron reagent on a cover slip, and place the cover slip over the section on a glass slide so that the tissue is bathed in the liquid.
3. Put in a refrigerator for 30 min. to aid in the crystallization of the nitrate.
4. Examine with polarized light under a microscope immediately on removal from refrigerator.

Result. The doubly refractive zones are due to the insoluble nitrate.

SULFHYDRYL AND DISULFIDE GROUPS

The earlier literature dealing with the application to tissue sections of the nitroprusside reaction for sulfhydryl groups, and the reduction of disulfide compounds to give this test, has been reviewed by Lison (1936, pages 133–136). The procedure given by Rapkine and recommended by Lison, as well as the methods of Bourne (1935) and of Hammett and Chapman (1938–1939), will be given. The latter investigators critically examined the nitroprusside test and concluded that it should not be considered a quantitative reaction; they established a well-defined procedure which they believe most likely to yield satisfactory qualitative results. However, the problem of diffusibility will no doubt limit, or eliminate, the use of any nitroprusside method.

Nitroprusside Test of Rapkine

SPECIAL REAGENTS

5% Sodium Nitroprusside. For plant tissues.
2% Sodium Nitroprusside. For animal tissues.
Ammonium Sulfate Crystals.
Conc. Ammonium Hydroxide.
10% Potassium Cyanide.
10% Trichloroacetic Acid.
5% Zinc Acetate.

PROCEDURE

A. *For free sulfhydryl groups*

1. Immerse the fresh tissue in the zinc acetate soln. for a few sec. This will stabilize the red color finally developed, as shown by Giroud and Bulliard (1933).

2. Add 1 drop of the sodium nitroprusside soln. to a section on a slide.

3. Add a crystal of ammonium sulfate and a drop of ammonium hydroxide.

Result. Sulfhydryl compounds such as glutathione produce a red color.

B. For total sulfhydryl groups

1. Treat sections of fresh tissue with 10% potassium cyanide for 5–10 min.

2.–4. Proceed with steps 1–3 in *A*.

C. For protein-bound sulfhydryl groups

1. Treat sections of fresh tissue with 10% trichloroacetic acid for 15 min. and wash thoroughly in water.

2.–4. Proceed with steps 1–3 in *A*.

NOTE: The diffusibility of the sulfhydryl compounds formed in *B*, or liberated in *C*, makes for particular unreliability in the localization of the groups in the sections.

Bourne Nitroprusside Test

SPECIAL REAGENTS

5% Acetic Acid.
5% Sodium Nitroprusside Saturated with Ammonium Sulfate.
Concentrated Ammonium Hydroxide.

PROCEDURE

1. Place fresh frozen sections of tissue in hot 5% acetic acid for 30–90 sec.

2. Pour off the acid and replace with nitroprusside-ammonium sulfate soln. for 2 min.

3. Add a few drops of ammonium hydroxide and examine at once.

Result. A purplish-blue color indicates sulfhydryl compounds.

Hammett and Chapman Nitroprusside Test

SPECIAL REAGENTS

27–29% Ammonium Hydroxide.

1% Sodium Nitroprusside.
Ammonium Sulfate Crystals.

PROCEDURE

1. Cover fresh tissue slice with 0.25 ml. water.
2. Add 0.05 ml. ammonium hydroxide and then 0.05 ml. of the nitroprusside soln.
3. Underline the tissue with 0.25 g. ammonium sulfate crystals and examine at once.

C. ORGANIC SUBSTANCES AND GROUPS

LIPIDS AND CHOLESTEROL*

By means of staining methods, it is impossible to distinguish with certainty between the various chemical types of the lipids with the possible exception of cholesterol and its esters. Until recently, the demonstration of lipids in general was usually carried out with Sudan dyes which dissolve in the lipids and color them. However, Jackson (1944) reported an improved method using acetic-carbol-Sudan III which he claims should supersede all other Sudan methods since it will bring out lipids that have been considered refractory to Sudan staining in the past. Jackson's paper includes an enlightening critical survey of previous work. To circumvent the loss of small fat globules from the tissue when alcohol or acetone dye solutions are used, Telford Govan (1944) employed Sudan dyes suspended in aqueous media. The Kay and Whitehead (1935) procedure using Sudan IV, the newer Jackson (1944) method employing acetic-carbol-Sudan III, and the Telford Govan (1944) technique will be described. The staining of lipids by means of fluorescent dyes according to Popper (page 105) would appear to have some advantages, particularly in the use of the water-soluble dyes such as Phosphine 3R.

Cholesterol and its esters may be visualized by the Liebermann-Burchardt reaction as adapted for histological use by Schultz (1924–1925), Romieu (1927), and Yamasaki (1931). The Schultz procedure has been employed more generally, and hence it will be given in detail. Lison (1936, page 210) has pointed out that, though the positive test is specific, a negative result does not necessarily

* See Bibliography Appendix, Ref. 3.

exclude the presence of cholesterol or its esters. The Windaus digitonin test for free cholesterol (Lison, 1936, pages 211–212) requires further investigation in the opinion of Kay and Whitehead in Lee's *Vade Mecum* (1937, page 281). By means of the polarizing microscope, cholesterol crystals can occasionally be observed in sections as birefringent rhombic plates. If the temperature is low enough, neutral fats and fatty acids can also be observed in some instances as birefringent crystals.

Kay and Whitehead Procedure for Sudan IV Stain for Lipids

SPECIAL REAGENTS

Stock Solution of Dye (can be used for at least 6 months). Prepare a saturated alcoholic solution by boiling 2 g. dye in 1 l. absolute alcohol; allow to cool.

Staining Solution (good for only about 4 hr. after being mixed). Add slowly, with stirring, to 7 vol. stock soln., 9 vol. of 45% alcohol. Filter after standing for 1 hr. The 45% alcohol is prepared by mixing 4 vol. absolute alcohol with 5 vol. distilled water.

PROCEDURE

1. Place formalin-fixed frozen sections in 50% alcohol for 5 min. in staining soln. for 30 min. at 37° (turn sections over after 15 min. for more even staining), in 50% alcohol several sec., and finally in distilled water a few min.
2. Pass through filtered hemalum and wash in alkaline tap water for several min.
3. Mount in glycerin jelly.

Result. The lipid will be stained red.

The sections should be stained the day after cutting since they tend to be sticky for a while just after the cutting. On the other hand, poor results are often encountered if the staining is delayed longer than one day after sectioning due, presumably, to crystallization of lipid material. The stain lasts for only a few months.

Jackson Procedure for Lipids Using Acetic-Carbol-Sudan III

SPECIAL REAGENTS

Sudan III Stock Solution. Cover 2 g. of the finely pulverized dye

with 450 ml. of 95% alcohol and heat on a water bath to simmering. Stir occasionally and then filter while hot. Transfer to a stoppered bottle and place in a refrigerator overnight. Filter while cold, and add distilled water dropwise from a burette, while stirring, to reduce the alcohol concentration to 80%. Allow to stand 24 hr.; filter and keep stoppered.

Acetic-Carbol-Sudan III Reagent. To a given volume of Sudan III stock soln., slowly add 5% phenol dropwise from a burette, stirring after each addition, to bring the alcohol concentration to 60% (*e.g.*, add 2 ml. phenol soln. to 6 ml. stock soln.). Let stand for several hr. keeping the bottle stoppered. Then, in the same dropwise manner, add glacial acetic acid in the proportion of 2.5 drops per ml. carbol-Sudan soln. After standing for 24 hr. in a stoppered bottle, filter the reagent. Do not use when the soln. is more than several days old.

5% Glacial Acetic Acid in 50% Alcohol.

PROCEDURE

1. Transfer formalin-fixed frozen sections to 50% alcohol for 1 min.
2. Place in the acetic-carbol-Sudan III reagent for 1.5 hr. or longer; keep vessel stoppered.
3. Differentiate in the acetic-alcohol soln. for 10–60 sec. and wash in distilled water for 1 min. In some cases it may be well to dilute the acetic-alcohol with more 50% alcohol.
4. A counterstain of recently filtered Delafield hematoxylin diluted with 2 vol. distilled water may be applied for 15 min., followed by differentiation in 0.5% hydrochloric acid until reddish (10–20 sec.), and treatment with very dilute ammonium hydroxide for 5 min. to develop the blue color.
5. Wash in distilled water and mount in glycerin jelly.

Result. The lipid will be stained red.

Telford Govan Technique for Sudan Dye Staining in Aqueous Media

SPECIAL REAGENTS

Sudan Dye Suspension. Add a saturated soln. of a Sudan dye in acetone dropwise from a capillary pipette to 1% gelatine soln.

containing 1% acetic acid until a deep brick-red color and a consistency of milk is obtained. Stir well during the addition. Hold the mixture at 37° for 2 hr. to evaporate the acetone or let stand in a warm room overnight. Filter off sediment through coarse paper.

1% Gelatin Solution.

PROCEDURE

1. Transfer frozen sections from water to 1% gelatin soln. for 2–3 min.
2. Stain for 30 min. at 37° with the suspension.
3. Wash sections in 1% gelatin soln. for 2–3 min.
4. Wash well in water.
5. Counterstain, if desired, and mount in glycerin jelly or Karo syrup.

Schultz Cholesterol Test

SPECIAL REAGENTS

2.5% Iron Alum $(NH_4)_2SO_4.Fe_2(SO_4)_3.24H_2O$.

Concentrated Sulfuric-Glacial Acetic Acid Mixture. Add the sulfuric acid slowly to an equal volume of glacial acetic acid, stirring and cooling the while. (Only the purest acids are suitable and the sulfuric must contain at least 98% sulfuric acid. The reagent is hydroscopic and must be protected from atmospheric moisture.)

PROCEDURE

1. Place formalin-fixed frozen sections in the iron alum solution for 3 days at 37°.
2. Rinse in distilled water, mount on slides and blot with filter paper.
3. Add a few drops of the acid mixture and cover with a cover-glass.

Result. A positive test for cholesterol, or its esters, is indicated by the appearance of a blue-green color which reaches its maximum intensity within a few min. Within 30 min. the sections acquire a brown discoloration. The appearance of large numbers of bubbles results from impure acids.

CAROTENE, CAROTENOIDS, AND VITAMIN A

The solubility of carotene, carotenoids, and vitamin A in organic solvents makes it necessary to employ frozen sections of tissue for histological studies on these substances. Unstained sections show yellow, orange, or brown regions due to the presence of constituents of this nature. The blue coloration given by concentrated sulfuric acid with these compounds has been employed by Steiger (1941) for the demonstration of carotene in leaves. The deep violet color developed in the presence of aqueous 1% iodine in 7% potassium iodide (Lison, 1936, page 245) is also characteristic of these polyenes, and when treated with oxidizing agents, such as chromic acid, they are bleached. Bourne (1935) adapted the Carr-Price reaction to tissue sections by placing frozen sections directly into a chloroform solution of antimony trichloride. It is well known that the blue color due to vitamin A fades very rapidly, while that due to carotene persists. As shown by Raoul and Meunier (1939), sterols produce a red color in the Carr-Price test. The detection of vitamin A in tissue by fluorescence is described on page 104.

Steiger Method for Carotene in Leaves

SPECIAL REAGENTS

Alkali-Alcohol Mixture. Combine 1 vol. saturated potassium hydroxide with 2 vol. of 40% alcohol and 3 vol. tap water.
Concentrated Sulfuric Acid.

PROCEDURE

1. Place green leaves in the alkali-alcohol mixture in a wide-mouth bottle and seal the glass stopper with vaseline.
2. After several days in the dark, when the fluid is green and the tissue yellow, transfer to distilled water for several hr.
3. Place small pieces of tissue on a slide and dry with filter paper.
4. Add 1 drop conc. sulfuric acid.

Result. Carotene is indicated by the appearance of dark blue crystals visible under the microscope. Grossly, a green color changing to blue can be observed.

RIBOFLAVIN

The detection of riboflavin in tissue sectiôns, based on reduction of the vitamin in acid medium to leucoflavin and reoxidation to bright red granules of rhodoflavin, was employed by Chèvremont and Comhaire (1939). Riboflavin can be recognized in tissue by its characteristic greenish-yellow fluorescence when irradiated with ultraviolet (page 104).

Chèvremont and Comhaire Method for Riboflavin

SPECIAL REAGENTS

Fixative. Formol-Nitron or formol-basic lead acetate soln.

Reductant Solution. Add zinc to hydrochloric acid to generate hydrogen.

Oxidant Solution. Dilute hydrogen peroxide.

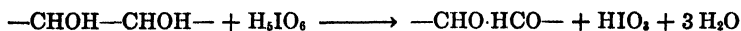
PROCEDURE

1. Fix tissue for 5 days and prepare sections.
2. Treat sections for 30 min. with reductant soln.
3. Rinse in water and treat with oxidant soln.
4. Examine under microscope.

Result. Bright red granules indicate presence of riboflavin. The localizations are probably unreliable due to the diffusibility of the riboflavin and its derivatives.

POLYSACCHARIDES IN GENERAL

A general reaction for the microscopic visualization of polysaccharides has been described by Hotchkiss (1946).* The reaction involves the oxidation of adjacent hydroxyl groups to aldehydes by means of periodate, and the coloring of the aldehyde with Feulgen reagent. The oxidation takes place according to the equation:

$$\text{—CHOH—CHOH—} + H_5IO_6 \longrightarrow \text{—CHO·HCO—} + HIO_3 + 3\,H_2O$$

The chief substances in plant tissues that show the stain are starches, cellulose, hemicelluloses, and pectins and, in animal tissues, glycogen, mucin, mucoproteins, and presumably hyaluronic acid and chitin. The pentoses of nucleic acid are so substituted that they

* Subsequent to this writing the author learned of the paper of McManus (1946) (Bibliography Appendix, Ref. 13) in which the same principal was independently presented.

will not give the reaction and cerebrosides, if present, would be expected to react.

Method of Hotchkiss for Polysaccharides

SPECIAL REAGENTS

Periodic Acid Solution A. Dissolve 400 mg. periodic acid (H_5IO_6, obtainable from *G. Frederick Smith Chemical Co.*) in 10 ml. distilled water, add 5 ml. *M*/5 sodium acetate and 35 ml. alcohol.

Periodic Acid Solution B. Dissolve 400 mg. periodic acid in 45 ml. distilled water and add 5 ml. *M*/5 sodium acetate.

Iodide-Thiosulfate Solution. Dissolve 1 g. potassium iodide and 1 g. sodium thiosulfate ($Na_2S_2O_3.5H_2O$) in 20 ml. distilled water and add with stirring 30 ml. alcohol followed by 0.5 ml. 2 *N* hydrochloric acid. A sulfur precipitate forms and settles out slowly although the soln. may be used immediately.

Feulgen Reagent. See page 67.

Sulfite Wash Solution. Add 0.5 ml. conc. hydrochloric acid and 2 ml. 10% potassium metabisulfite to 50 ml. distilled water.

PROCEDURE

1. If water-soluble polysaccharides are to be stained, fix the tissue in a dehydrating soln. such as Carnoy fluid (page 45). Otherwise, use any of the usual fixatives. After fixation remove traces of mercury, if present, with iodine and be sure any formaldehyde is completely removed by washing. (70% alcohol may be used as a washing soln. if it is desired to avoid removal of water-soluble polysaccharides.)
2. Place section or smear in the periodic acid soln. A or B (depending on whether an alcoholic or aqueous soln. is desired) for 5 min.
3. Flood with 70% alcohol (or water) and transfer to the iodide-thiosulfate soln. for 5 min.
4. Again wash with 70% alcohol (or water) and then transfer to the Feulgen reagent for 15–45 min.
5. Rinse with the sulfite wash soln., dehydrate, and mount as usual.

Result. Polysaccharides are indicated by the violet fuchsin color.

NOTE: If free tissue aldehydes are present it is necessary to remove them first as on page 93.

If the periodate and iodate are not washed out they will give rise to a brownish coloration in the Feulgen reagent.

Control sections or smears may be made by placing in 70% alcohol (or water), instead of in the periodic acid soln. A or B, and then carrying through the remaining steps without change.

Egg albumin adhesive may take a slight stain due to the carbohydrate content of this material.

If neutral polysaccharides are to be stained, a counterstain with a basic dye (such as 0.02 mg. malachite green per ml. water) may be used. If mucin or acid polysaccharides are to be stained, the counterstain should be an acid dye.

ACID POLYSACCHARIDES — HYALURONIC ACID*

For the demonstration of acid polysaccharides of the hyaluronic acid type Hale (1946) employed fixation in a dehydrating medium to prevent solution of the water-soluble acid polysaccharide, combination of the latter with iron, and demonstration of the iron by means of the Prussian blue reaction. The iron will not combine with neutral polysaccharides or proteins according to Hale. In order to differentiate between hyaluronic acid and other substances which might give the blue stain, Hale suggests that hyaluronidase be used to digest away the hyaluronic acid in the section on one slide and a comparison be made to an undigested section.

Hale Method for Acid Polysaccharides

SPECIAL REAGENTS

Acetic-Iron Solution. Mix equal vol. dialyzed ferric hydroxide, concentration not stated (Hale used the product of the *British Drug Houses Ltd.*), and 2 *M* acetic acid.

0.02 M Potassium Ferrocyanide in 0.14 M Hydrochloric Acid.

PROCEDURE

1. Fix 3–4 mm. pieces of tissue in Carnoy fluid (6 vol. absolute alcohol, 3 vol. chloroform, and 1 vol. glacial acetic acid) for 0.5 hr.
2. Treat with absolute alcohol; clear, and mount in paraffin.
3. Prepare sections and place on slides without albumin adhesive.
4. Flood the deparaffinized sections with the acetic-iron soln., and after 10 min. wash well with distilled water.
5. Treat the sections with the ferrocyanide soln. for 10 min. and

* See page 46 for the staining of mucoproteins.

wash with water and counterstain if desired (it is well to use a red counterstain such as fuchsin).

6. Rapidly dehydrate, clear in xylol, and mount in Canada balsam.

Result. Acid polysaccharide is indicated by the blue color.

MUCOPROTEINS*

Toluidene blue will stain quite a variety of acid substances, but the metachromatic staining by this dye of mucoid compounds containing polysaccharide esters of sulfuric acid is specific for these compounds, provided that the method of Lison (1935) is strictly adhered to (Sylvén, 1941, 1945). Sylvén (1941) has made a thorough study of the staining and he emphasized that "false" metachromatic staining can be obviated by the prompt removal of water by alcohol after the staining, the alcohol assuring a "true" reaction which is the red stain characteristic of, and specific for, the polysaccharide sulfates in tissue. Holmgren and Wilander (1937) found that basic lead acetate solution was a superior fixative for tissues to be subjected to the metachromatic toluidene blue stain, but Sylvén now employs a mixture of this fixative with formalin to reduce the time required for the fixation. The staining time can be reduced by aging the dye solution, and the greater the alcohol concentration in the dye solution the paler the resulting stain will be. In order to bring out mast cell granules properly, the dye is made up in alcohol of a concentration of 30% or higher (Sylvén).

According to the claim of Hempelmann (1940), chondroitin and mucoitin sulfuric acid proteins can be differentiated from one another in histological preparations by means of the toluidene blue stain. In a dilution of 1 : 1,280,000 an aqueous solution of toluidene blue is supposed to stain the chondroitin material in paraffin sections a violet-red color, while the mucoitin protein complex remains unstained. Differentiation is also claimed when the dye is used in a 1 : 410,000 dilution in a solution of 10 vol. alcohol and 45 vol. water. The alcohol concentration is stated to be critical, presumably both mucoproteins will be stained if the proportion of alcohol is less, and neither if it is greater. No confirmation of these claims has been made; in fact, to the writer's knowledge several attempts to do so have failed.

*** See Bibliography Appendix, Refs. 8 and 10.**

A fundamental study of metachromasy of basic dyes has been published by Michaelis and Granick (1945).

See page 45 for another method of staining acid polysaccharides, and page 50 for the staining of mucin.

Lison Method for Polysaccharide Sulfate Compounds (after Sylvén)

SPECIAL REAGENTS

Fixing Solution. Mix equal vol. of 8% basic lead acetate soln. and 14–16% formalin.

Toluidene Blue Solution. Prepare separately (*a*) 0.1% dye in 1% alcohol and (*b*) 0.1% dye in 30% alcohol, and let stand for a number of days to age.

PROCEDURE

1. Fix the tissue for 12–24 hr. in the fixing soln.
2. Prepare paraffin sections in the usual manner.
3. Stain the sections for 30 min. using soln. *a* on some, and soln. *b* on others. Soln. *a* gives a more intense stain.
4. Wash well in alcohol briefly, immediately after removing from the dye soln.
5. Mount in natural cedar oil.

GLYCOGEN AND MUCIN*

A critical comparison of the iodine, Best carmine, and Bauer-Feulgen methods for demonstrating glycogen microscopically was made by Bensley (1939), who concluded that the Bauer-Feulgen method, which depends on the reaction of the aldehyde groups in the carbohydrate with the reagent, is by far the best if the tissue is promptly fixed in alcohol-formalin solution. When chrome salts are present in the fixative, the visualization of intracellular glycogen was found to require the Best carmine stain since the Bauer-Feulgen method is not specific in those cases, and the iodine technique is not suited for high-power studies. A procedure for preparing paraffin sections for the carmine stain was given by Mullen (1944), who employed celloidin to hold the deparaffinized sections to the glass slide.

Mitchell and Wislocki (1944) reported that the ammoniacal silver

* See Bibliography Appendix, Refs. 2 and 12.

nitrate method which Pap (1929) employed for the staining of reticulum visualized glycogen more intensely and consistently than either the Best carmine or Bauer-Feulgen procedures. The admitted drawback to this method is the fact that since reticulum fibers of connective tissue are also stained it cannot be applied to this tissue. However, the authors feel that in other cases the ammoniacal silver nitrate method has advantages over those previously employed. Gomori (1946) subsequently modified this procedure and developed a more selective method which demonstrates glycogen and mucin, but eliminates possible interference by desoxyribonucleic acid, uric acid, and granules of enterochromaffin cells, all of which can reduce silver solutions under certain conditions. However, melanin is stained, and except for this the method enables the same localizations of the reducing substances as the Bauer-Feulgen procedure. Should insoluble calcium salts be present they too will stain black. A preliminary 10 min. treatment of the sections with citrate buffer of *p*H 3–4 will remove calcium deposits.

The differentiation of glycogen from other substances that give positive reactions may be made in some instances by employing saliva to digest away the glycogen selectively. See page 46 for the staining of mucoproteins.

Bauer-Feulgen Stain for Glycogen (after Bensley)

SPECIAL REAGENTS

Alcohol-Formalin Fixative. 9 vol. absolute alcohol plus 1 vol. neutral formalin. The alcohol may be first saturated with picric acid.

*Feulgen Reagent.** Heat to dissolve 1 g. basic fuchsin in 100 ml. distilled water. Filter while warm, cool, add 20 ml. 1 *N* hydrochloric acid and 1 g. sodium bisulfite. Let stand 24 hr. The soln. should be straw colored.

1% or 4% Chromic Acid.

Bisulfite Rinsing Solution. 1 vol., 1 *M* sodium bisulfite plus 19 vol. tap water.

PROCEDURE

1. Fix very small pieces (2–3 mm.) of fresh tissue in the alcohol-

*See pages 65 and 67 for other methods of preparing this reagent.

formol solution for 24 hr. (Deane, Nesbett, and Hastings, 1946, recommend the use of ice-cold alcohol-picric acid-formalin to preserve the glycogen throughout the tissue block.) Wash in absolute alcohol and embed in paraffin, being careful to prevent overheating. (It is essential that very fresh tissue be used since glycogen is rapidly autolyzed. The Altmann-Gersh freezing and drying technique for fixation may also be used; in fact it can lead to a truer picture of the glycogen distribution, as shown by Bensley and Gersh, 1933a).

2. Section, mount on slides, and deparaffinize as usual.
3. Place in 4% chromic acid 1 hr. or in the 1% soln. overnight.
4. Wash in running water for 5 min., place in Feulgen reagent 10–15 min., rinse with three changes of bisulfite soln. for 1.5 min. in each change, and wash in running water for 10 min.
5. Nuclei may be counterstained with hematoxylin.
6. Dehydrate, clear, and mount in balsam.
7. As a negative control, remove the glycogen from some of the sections, brought down to water, by adding fresh saliva. During a 15–30 min. period, change the saliva several times. Wash with water at 37° to remove mucus and stain as above beginning with step 3. Comparison of these sections with those not given the saliva treatment helps to distinguish the glycogen regions.

Result. The glycogen appears deep red-violet, the nuclei lavender.

Best Carmine Stain for Glycogen (after Bensley)

SPECIAL REAGENTS

Alcohol-Formalin Fixative. Same as the reagent for Bauer-Feulgen stain.

Carmine Stain Stock Solution. Gently boil 2 g. carmine, 1 g. potassium carbonate, and 5 g. potassium chloride in 60 ml. distilled water until color darkens. After cooling, add 20 ml. conc. ammonia and let stand 24 hr. This solution may deteriorate in a month in a warm room; keep well stoppered.

Fresh Carmine Stain. 10 ml. stock soln., 15 ml. conc. ammonia, and 30 ml. methanol (C. P.).

PROCEDURE

1. Follow steps 1 and 2 for Bauer-Feulgen stain.
2. After bringing down to distilled water, stain nuclei with hematoxylin.
3. Transfer to fresh carmine stain and after 20 min. wash in three changes of methanol, dehydrate in acetone, clear in toluol, and mount in balsam.
4. Run negative control sections as in step 7 for the Bauer-Feulgen method but apply the carmine stain above.

Result. The glycogen will appear brilliantly red.

Gomori Procedure for Glycogen and Mucin

SPECIAL REAGENTS

Fixative. One of the alcohol fixatives such as alcohol-picric acid-formalin (page 48) for glycogen. Any routine fixative for mucin.

0.5% Collodion in Alcohol-Ether Solution.

5% Chromic Acid.

1–2% Sodium Bisulfite.

Silver-Methenamine Stock Solution. Add 5 ml. 5% silver nitrate soln. to 100 ml. 3% methenamine (hexamethylenetetramine) soln. Shake until the initial heavy white precipitate disappears, and store in refrigerator.

Alkalized Silver-Methenamine Solution. To 25 ml. silver-methenamine stock soln. add 25 ml. distilled water and 1–2 ml. 5% borax ($Na_2B_4O_7.10H_2O$).

0.1% Gold Chloride.

2–3% Sodium Hyposulfite.

PROCEDURE

1. Fix tissue and prepare paraffin sections as usual.
2. Run sections through xylol, alcohols, and water. (For glycogen, protect sections on slides by dipping into collodion soln. before transferring to the final alcohol soln.)
3. Place slides in 5% chromic acid for 1—1.5 hr.
4. Wash in running tap water for 10 min. and treat with the bisulfite soln. for 1 min. to remove remaining traces of chromic acid.
5. Wash in running tap water for 5 min., rinse in distilled water, and incubate at 37–45° in the alkalized silver-methenamine soln. Examine sections under microscope every 15 min. Staining is com-

pleted when the glycogen and mucin appear deep brown or black. The background will be yellowish. Usually 1–3 hr. is required.

6. Rinse well in repeated changes of distilled water and tone in the gold chloride soln. for 5 min.
7. Rinse in distilled water and then in the hyposulfite soln. to remove unreacted silver.
8. Wash in tap water and counterstain if desired.
9. Mount as usual.

Result. Glycogen and mucin will appear in shades from grey-brown to black on an unstained background. Sometimes the collodion film becomes stained and it can be removed by acetone or alcohol-ether.

STARCH

The common practice of employing a dilute iodine solution to develop a blue color with starch can be applied to sections of plant material, as can the crystal violet stain followed by washing with saturated picric acid solution. The use of formaldehyde as a swelling agent to obtain special effects with safranine O and fast green FCF was described by Bates (1942). Starch granules can also be recognized by the characteristic black crosses they exhibit due to their doubly refractive properties when viewed under the microscope with polarized light.

The following procedure of Milovidov (1928) is well suited for the preparation of permanently mounted sections stained for starch.

Milovidov Method for Starch

SPECIAL REAGENTS

Aniline Fuchsin Stain.
5% Alcoholic Aurantia.
2% Tannin.
1% Toluidine Blue, Gentian Violet, or Methyl Green.

PROCEDURE

1. Fix plant tissue in Regaud fluid and prepare sections as usual.
2. Stain sections with aniline fuchsin for 5 min. and differentiate in the aurantia soln.

3. After washing sections in distilled water, mordant for 20 min. in the tannin soln. and again wash.

4. Stain sections in either toluidine blue, gentian violet, or methyl green for 5–10 min.

5. Differentiate in 95% alcohol, dehydrate in absolute alcohol, clear in xylol, and mount in balsam.

Result. The starch will appear as either blue, violet, or green granules depending on which of the stains was used in step 4. The mitochondria will appear red.

CELLULOSE

Post and Laudermilk (1942) Iodine Stain for Cellulose

SPECIAL REAGENTS

Iodine Solution. 20 ml. 2% iodine in 5% potassium iodide, 180 ml. distilled water, and 0.5 ml. glycerol.

Lithium Chloride Solution. Saturate 15 ml. distilled water at 80°, cool; use supernatant soln.

PROCEDURE

1. Tease out sections or fibers.

2. Apply 2–3 drops of the iodine soln. and after 10 sec. blot with filter paper and dry.

3. Add a drop of the lithium chloride soln., cover, and examine.

Result. Cellulose appears in the following colors depending on its source:

Typical color	Fiber
Light blue	cotton, soda pulp, bleached sulfite, straw, esparto
Dark blue	pineapple fiber
Greenish blue	linen
Green to yellowish green	sisal, Manila hemp, yucca
Yellow	yucca, ground wood, hemp, Manila hemp
Lemon yellow	kapok
Brownish yellow	jute

CHITIN*

The horny carbohydrate material, chitin, requires special treatment to soften it sufficiently for the preparation of paraffin sections.

* See Bibliography Appendix, Ref. 16.

The most recent method for this treatment is that of Murray (1937), but the Diaphanol technique has been widely employed. Once sections are prepared they may be stained by the procedure of Zander, Schulze, or Bethe given in Lee (1937, page 600).

Murray Method for Softening Chitin

SPECIAL REAGENTS

Formalin-Saline Fixative. 10% formalin in 0.8% sodium chloride soln.

Dehydrating Fixative. Equal vol. absolute alcohol, chloroform, and glacial acetic acid to which mercuric chloride is added to saturation (about 4%).

Chloral Hydrate-Phenol Reagent. Equal weights of chloral hydrate and phenol warmed together until they blend to an oily liquid that is fluid at room temperature.

PROCEDURE

1. Fix material in the formalin-saline soln.
2. Transfer to the dehydrating fixative.
3. Place specimen in the chloral hydrate-phenol reagent for 12–24 hr. or longer.
4. Clear with xylol, chloroform, or carbon disufide and imbed in paraffin.

Diaphanol Method for Softening Chitin

SPECIAL REAGENTS

Diaphanol Solution. Pass vapors of chlorine dioxide into ice-cold 50% acetic acid. Store in a cool dark place in a glass-stoppered bottle. (Before the war, Diaphanol was sold by Leitz; and Lee—1937, page 598—recommends buying, rather than preparing, the soln. However, it will probably be impossible to buy for some time, and there is no reason why the reagent cannot be safely prepared if the obvious precautions of working in a hood, etc., are taken.)

PROCEDURE

1. Fixed material is rinsed in 63% alcohol and placed in Diaphanol in a glass-stoppered bottle in diffuse daylight until bleached

and softened. The specimen should be pierced or cut to allow escape of carbon dioxide.

2. If the Diaphanol becomes discolored, transfer to a fresh portion of the soln.

3. Place in 63% alcohol until hardened and then pass through tetralin into paraffin.

Methods for Staining Chitin

The softened material, or sections of it, may be tested for chitin by a variety of color reactions. Zander treated for a short time with a drop of fresh iodine in potassium iodide soln., followed by a drop of strong zinc chloride soln. Upon removal of the reagents with water, a violet color is obtained in the presence of chitin. Schulze divided the material into two portions. One was subjected to the procedure of Zander and the other was treated with iodine and then conc. sulfuric acid. The latter test serves to distinguish chitin from cellulose and tunicin since chitin yields a brown color while the others give a blue. Bethe employed freshly prepared 10% aniline hydrochloride containing a drop of conc. hydrochloric acid for each 10 ml. After sections were placed in this soln. for 3–4 min., they were rinsed with water and the slides were then placed, sections downward, in a bath of 10% potassium dichromate. Chitin produces a green coloration which becomes blue in tap water or ammoniacal alcohol.

ASCORBIC ACID

The stain for ascorbic acid was developed in 1933 by Bourne, who utilized the fact that reduced silver is deposited when ascorbic acid in tissue interacts with acid silver nitrate. Bourne (1936) published a critical survey of this stain and his recommended procedure is given below with a subsequent modification by Barnett and Bourne (1941) designed to increase the specificity of the test by dissolving precipitated silver salts in dilute ammonia. Giroud and Leblond (1936) also investigated the technique and its applications, and in reply to criticisms of the specificity of the stain, these authors (1937) point out that the positive test is specific for ascorbic acid but a negative result does not necessarily mean that

ascorbic acid is absent. Tonutti (1938) washed the tissue in 5.4% levulose solution before staining in order to remove blood. The reliability of the localizations obtained with the silver stain remains to be proved, according to Danielli (1946a). It would be necessary to establish that the ascorbic acid is attached to a nondiffusible body and that the reaction product could not diffuse, or that the ascorbic acid site has a high affinity for the reaction product.

Bourne Silver Stain for Ascorbic Acid

I. Reduced Ascorbic Acid

SPECIAL REAGENTS

Acid Silver Nitrate. Add 5 ml. glacial acetic acid to 100 ml. 5% silver nitrate.

5% Ammonium Hydroxide.

PROCEDURE

1. Place frozen sections of fresh tissue in the acid silver nitrate soln. for a few minutes, and then treat with 5% ammonium hydroxide.
2. Wash with distilled water. If desired, lipids can be then stained with a Sudan dye in 90% alcohol.
3. After clearing, mount in glycerin.

Result. Granules containing reduced ascorbic acid appear black.

II. Reduced and Oxidized Ascorbic Acid

PROCEDURE

1. Expose the fresh tissue to the vapor of glacial acetic acid for several minutes.
2. Cut into thin pieces and subject to an atmosphere of hydrogen sulfide for 15 min. in order to reduce the oxidized form.
3. Remove hydrogen sulfide by placing in a vacuum for 10–30 min. followed by a good stream of nitrogen gas for 15 min.
4. Treat with acid silver nitrate soln. followed by ammonium hydroxide as above.

Should glutathione be present in quantities sufficient to inhibit the test, wash the tissue momentarily after the hydrogen sulfide treatment and immerse at once into a mercuric acetate soln. for a few minutes. After washing, apply the acid silver nitrate solution and then the ammonium hydroxide.

PROTEIN REACTIONS

Many of the tests for proteins are poorly adapted to histochemical work because the strong acid or alkali that they require has too great a disintegrative effect on the cellular structure. Tests which particularly fall into this group are the biuret reaction for component peptides, the xanthoproteic reaction for phenolic constituents, Millon's tyrosine reaction, Romieu's tryptophane test, the tryptophane reaction of Voisenet-Fürth, and the diazo reaction for histidine and tyrosine. Serra's arginine test, which is much less drastic, and Berg's ninhydrin reaction for α-amino acid groups, which uses no corrosive reagents although heating is required, will both be described as well as two of the previous group, Millon and Romieu reactions.

ARGININE AND ARGININE-CONTAINING PROTEINS

In another of those coincidences that occasionally turn up, Serra at the University of Coimbra, Portugal, and Thomas at the University of Missouri, independently, and without knowledge of the other's work, adapted the Sakaguchi (1925) reaction for arginine to its histochemical identification. The reaction is based on the development of an orange-red color with arginine when α-naphthol and hypobromite or hypochlorite react with it in an alkaline medium.

The first description of the method by Serra (1944a,b) was followed by a report of Serra and Queiroz Lopes (1944), who emphasized the usefulness of the reaction for the visualization of the basic proteins such as those contained in cell nuclei. Subsequently, Serra (1946) summarized the work of his group on the arginine reaction in the course of a more general article dealing with histochemical tests for proteins and amino acids. Serra pointed out that a positive reaction is found only with arginine and the rather rare compounds glycocyamine, gelegine, and agmatine, negative reactions being given by guanidine, urea, ornithine, creatine, creatinine, asparagine, histidine, and other amino acids. The reaction is specific for guanidine derivatives in which one hydrogen atom of one or both amino groups is substituted by an alkyl, fatty acid, or cyano radical. Substitution of other radicals has not been tested, while guanidine derivatives in which both hydrogen atoms of one amino group are substituted do not give the color (Thomas, 1946).

The procedures of Serra and Thomas differ in certain details and, at the date of this writing, no comparison of the two has been made. An advantage of the method of Thomas is that it does not employ cooling in an ice bath during the reaction because of the substitution of hypochlorite for hypobromite. Serra mounts his sections in glycerol after several transfers through this medium and he has reported that in this fashion the color, otherwise stable for only a short time, is stabilized for months.

Serra Method for Arginine and Arginine-Containing Proteins

SPECIAL REAGENTS

Acetic-Alcohol-Formalin Fixative. Add a few drops of glacial acetic acid to each 10 ml. of a mixture of 2 vol. 96% alcohol and 1 vol. formalin.

1% α-Naphthol in 96% Alcohol. Store in a refrigerator. Dilute 1:10 with 40% alcohol before use.

4% Sodium Hydroxide.

2% Sodium Hypobromite. With stirring and cooling, add 2 g. or approximately 0.7 ml. bromine to 100 ml. 5% sodium hydroxide. Store in a refrigerator.

40% Urea.

PROCEDURE

1. Fix the material in the acetic-alcohol-formalin mixture. Wash well in water.
2. Transfer to a watch glass kept at 0–5° in an ice bath, and treat for 15 min. at this temperature with a mixture of 0.5 ml. α-naphthol soln., 0.5 ml. 1 *N* sodium hydroxide, and 0.2 ml. 40% urea.
3. Add 2 ml. 2% hypobromite, and after 3 min. stir in 0.2 ml. urea soln. and then 0.2 ml. of the hypobromite. The maximum color develops in 3–5 min.; intensify it by a subsequent treatment with the hypobromite for 3 min.
4. Pass through four glycerol baths, leaving for 2–3 min. in each. In glycerol the color is stable for months even at room temperature. The fading is inhibited by storage in the cold.

Result. An orange-red color characterizes a positive reaction.

Thomas Method for Arginine and Arginine-Containing Proteins

SPECIAL REAGENTS

0.1% α-Naphthol in 10% (by vol.) Ethyl Alcohol.

0.15 N Sodium Hyprochlorite in 0.05 N Sodium Hydroxide. For preparation of the hypochlorite see page 239; or prepare from Clorox which is standardized and then stored at 3–5° in a dark bottle. Dilute the hypochlorite to the proper strength each time before use. Clorox is approximately 1.6 *N*; standardize by adding 1 ml. Clorox to 5 ml. 1 *N* potassium iodide, 8 ml. conc. hydrochloric acid (sp. gr. 1.19), and 45 ml. water. Titrate with 0.1 *N* sodium thiosulfate using starch indicator.

20% Urea in 0.05 N Sodium Hydroxide.

Tertiary Butyl Alcohol Solution. Add 1 ml. 5 *N* sodium hydroxide and 19 ml. water to 80 ml. of the tertiary butyl alcohol. Shake well and let stand; an aqueous layer collects on the bottom of the vessel.

Pure Tertiary Butyl Alcohol.

Aniline.

Toluene.

PROCEDURE

1. Fix animal tissues in Bouin fluid (75 ml. saturated picric acid soln., 25 ml. formalin, 5 ml. glacial acetic acid). Onion root tips were treated with medium chrome-acetic fixative.
2. Prepare paraffin sections in the usual manner. Do not remove paraffin from sections until test is to be applied.
3. Place slide with sections in the α-naphthol soln. for at least 3 min.
4. Transfer to each of the following solns. in succession for the periods indicated: hypochlorite, 20 sec.; urea, 5 sec.; 80% tertiary butyl alcohol, 30 sec.; pure tertiary butyl alcohol, 2 min.; aniline, 2 min.; toluene, 5 sec.; and finally mount in Clarite. Use a stop watch to time the immersions in each fluid.

TRYPTOPHANE IN PROTEINS

The red or violet color formed with proteins in the presence of phosphoric acid is the basis of the Romieu reaction. Blanchetière

and Romieu (1931) presented evidence that the effect was the result of tryptophane groups in the protein. As in the other protein tests, the drastic nature of the reaction seriously interferes with its use in most instances, as does the diffusibility of the color formed.

Romieu Reaction for Tryptophane in Proteins

SPECIAL REAGENTS

Syrupy Phosphoric Acid.

PROCEDURE

1. Fix tissue in alcohol, formalin, or Bouin fluid.
2. Prepare fairly thick paraffin or celloidin sections and remove the infiltrating agent.
3. Place a drop of the phosphoric acid on a section and set in an oven at 56° for a few min.
4. Examine on removal from oven.

Result. A positive test is manifest by the formation of a red or violet color.

TYROSINE IN PROTEINS

Bensley's histochemical adaptation of well-known Millon reaction for proteins containing tyrosine has been employed in studies by Bensley and Gersh (1933b).

Millon Reaction for Tyrosine in Proteins (after Bensley and Gersh)

SPECIAL REAGENTS

Millon Reagent. Add 1 vol. 40% nitric acid (add 600 ml. distilled water to 400 ml. conc. nitric acid, sp. gr. 1.42; let stand for 48 hr.) to 9 vol. distilled water and saturate with mercuric nitrate crystals by frequent shaking over several days. Filter, and, to 400 ml. of filtrate, add 3 ml. 40% nitric acid and 1.4 g. sodium nitrite.

1% Nitric Acid.

PROCEDURE

1. Mount sections on slides without using water. The freezing-drying technique is preferable.
2. Place in cold Millon reagent. Since the maximum color is developed in about 3 hr., remove each slide at a different time, dip

immediately in 1% nitric acid and dehydrate rapidly in absolute alcohol.

3. Clear in xylol and mount in balsam.

Result. A brick-red or rose color develops in the presence of tyrosine or proteins containing tyrosine.

α-AMINO ACID GROUPS IN PROTEINS

Less soluble peptides and proteins containing α-amino acids may be demonstrated at their loci in tissue sections by either the alloxan or ninhydrin reactions. A tendency for the color to diffuse in the alloxan reaction indicates that caution should be applied in interpreting the test, as Giroud (1929) has warned; furthermore the specificity is not great enough to exclude the need for confirmatory tests (Romieu, 1925). Hence only the ninhydrin reaction of Berg (1926) will be described. A positive reaction is obtained with many amines, aldehydes, and ammonium compounds as well as with the amino acids, but the solubility of these compounds enables their easy removal as a rule.

Berg Ninhydrin Test for α-Amino Acid Groups

SPECIAL REAGENTS

0.2% Ninhydrin.

PROCEDURE

1. Fix tissue in 10% formalin.
2. Wash in water and prepare frozen sections.
3. Boil sections in 2 ml. of the ninhydrin soln. for 1 min.
4. Wash in water and mount in glycerin or glycerin jelly.

Result. α-Amino acid groups give rise to an intense blue or violet color; this should be observed the same day as it fades rapidly.

NOTE: Serra and Quieroz Lopes (1945) employed a mixture of equal vol. of 0.4% ninhydrin in distilled water and phosphate buffer of *p*H 6.98 (6 ml. *M*/15 secondary sodium phosphate—11.1876 g. $Na_2HPO_4.2H_2O$ per liter—and 4 ml. *M*/15 primary potassium phosphate—9.078 g. KH_2PO_4 per liter). They heat the sections in the liquid in a watch glass placed over a boiling water bath. For cementing of the preparations they employ the mixture of Romeis, which is 80 g. colophonium and 20 g. carefully heated lanolin.

MELANIN

Perhaps the most characteristic microchemical test for melanin is its ability to reduce ammoniacal silver nitrate. Of course many other tissue constituents have this property so that the test is of value only when possible interferences (page 48) are considered. Dublin (1943) applied the Bodian silver method to the demonstration of melanin; his procedure follows.

Dublin Application of the Bodian Method to Demonstration of Melanin

SPECIAL REAGENTS

Protargol Solution. Prepare fresh each time by adding one or more drops of Protargol (*Winthrop*)—other brands do not appear to be satisfactory—to water in a staining jar. The color should be light amber. *Do not stir or mix the solution since this results in gumming.*

1.0% Hydroquinone.

0.5% Auric Chloride.

5.0% Oxalic Acid.

10% Sodium Thiosulfate.

PROCEDURE

1. Fix tissue in 10% formalin.
2. Prepare paraffin sections 8 μ thick.
3. Treat the deparaffinized sections, after passing through graded alcohols to water, with the Protargol soln. overnight.
4. Rinse with water and place in the hydroquinone soln. for 10 min.
5. Rinse with water and place in the auric chloride soln. for 5 min.
6. Rinse with water and place in the oxalic acid soln. for 5 min.
7. Rinse with water and place in the thiosulfate soln. for 5 min.
8. Wash in running tap water for 10 min.
9. Dehydrate, clear, and mount as usual.

Result. The melanin will appear black and the background a purplish brown or gray.

HEMOGLOBIN

Of the many tests for hemoglobin in tissue, smears and blood cells the more recent procedures of Ralph (1941), Goulliart (1939, 1941), and Dunn (1946) will be given. Previously Dunn and Thompson (1945) had modified the Van Gieson stain, and later these authors (1946) adapted the patent blue method of Lison (1938) for the staining of hemoglobin. The cyanol method of Dunn given below is a simplification of the technique of Fautrez and Lambert (1937).

Ralph Method for Hemoglobin

SPECIAL REAGENTS

Benzidine Reagent. 1% benzidine in absolute methanol.
Peroxide Reagent. 25% Superoxol in 70% ethanol.

PROCEDURE

1. Flood the dried blood or tissue smear on a glass slide with the benzidine reagent for 1 min.
2. Drain off and flood the smear with the peroxide reagent for 1.5 min.
3. Wash in distilled water for 15 sec.
4. Dry and mount in neutral balsam.

Result. Hemoglobin will appear dark brown.

Goulliart Method for Hemoglobin

SPECIAL REAGENTS

Glacial Acetic Acid Containing a Few Crystals of Potassium Iodide. Do not use after a week.

PROCEDURE

1. Treat a dried smear or frozen section on a slide with a drop of reagent.
2. Examine after 30 min. with a polarizing microscope for groups of very small boat-shaped birefringent crystals of protoiodoheme. These crystals slowly change into square tabular Teichmann crystals. The reaction may be speeded by warming.

Dunn Method for Hemoglobin

SPECIAL REAGENTS

Cyanol Stock Solution. Dissolve 1 g. cyanol (*National Aniline Division, Allied Chemical and Dye Corp.*) in 100 ml. distilled water, add 10 g. pure zinc powder, and 2 ml. glacial acetic acid. Bring mixture to a boil and the blue color will soon fade out. The soln. is stable for several weeks.

Cyanol Working Solution. Just before use filter 10 ml. of the stock soln., add 2 ml. glacial acetic acid and 1 ml. commercial 3% hydrogen peroxide.

PROCEDURE

1. Prepare frozen or paraffin sections of tissue fixed in 4% formaldehyde buffered to *p*H 7.0.
2. Bring sections to water and stain in cyanol working solution 3–5 min.
3. Rinse in water and counterstain in safranin (1:1000 in 1% acetic acid) 1 min.
4. Wash in water, dehydrate, clear, and mount in Clarite.

Result. Hemoglobin stains dark blue to bluish-gray; nuclei, red; and cytoplasm, light pink.

BILE PIGMENTS AND ACIDS

The well-known Gmelin test has been adapted to the microscopic detection of bile pigments by simply adding a drop of nitric acid containing some nitrous acid to the sample on a slide. A positive test is indicated by the appearance of a green color changing to red and finally to blue. Stein's test (1935), given below, is probably more satisfactory. Bile salts and acids may be precipitated by barium and the precipitate stained with acid fuchsin according to the technique of Forsgren (1928).

Stein Test for Bile Pigments

SPECIAL REAGENTS

Iodine Reagent. 2 or 3 vol. Lugol solution (6 g. potassium iodide and 4 g. iodine dissolved in 100 ml. distilled water) plus 1 vol. tincture of iodine.

5% Sodium Hyposulfite.

PROCEDURE

1. Fix for a short period in alcohol or 10% formalin.
2. Prepare paraffin sections and employ egg albumin to hold to slides.
3. After removal of paraffin and bringing down to water, subject sections to the iodine reagent for 6–12 hr.
4. Wash in distilled water and decolorize with the sodium hyposulfite for 15–30 sec.
5. Wash in distilled water and stain with alum carmine for 1–3 hr.
6. Wash in distilled water, dehydrate in acetone, clear in xylol, and mount in balsam.

Result. Bile pigments appear emerald green. Localizations cannot be considered reliable due to the diffusibility of the reactants and the final color.

Forsgren Test for Bile Acids

SPECIAL REAGENTS

3% Barium Chloride.
0.1% Acid Fuchsin.
1% Phosphomolybdic Acid.
Aniline Blue-Orange G Stain. Dissolve 0.5 g. aniline blue, 2 g. orange G, and 2 g. oxalic acid in 100 ml. distilled water.

PROCEDURE

1. Treat small pieces of tissue for 6–12 hr. with the barium chloride soln.
2. Fix in 10% formalin for 12–18 hr.
3. Prepare paraffin sections.
4. Stain sections for 1–3 min. in the acid fuchsin soln., and wash in distilled water.
5. Place sections in the phosphomolybdic acid soln. for 0.5–1.0 min. and wash in distilled water.
6. Treat sections for 3–5 min. with the aniline blue-orange G stain and wash in distilled water.
7. Dehydrate, clear, and mount in balsam.

Result. Bile secretory granules appear reddish.

ALDEHYDES, NUCLEIC ACIDS, AND "PLASMAL"

The research of Feulgen and co-workers (1924,1938,1939) and Imhäuser (1927) led to the demonstration of a loosely bound aldehyde, "plasmal," in animal tissues. The bound form, "plasmalogen," liberates "plasmal" when treated with mercuric chloride or subjected to prolonged acid hydrolysis. The Feulgen reaction, which depends on the formation of a purple-colored compound when aldehydes react with fuchsin-sulfurous acid, is also given by desoxyribonucleic acid (thymonucleic acid) after its purine bases are removed by acid hydrolysis, but ribonucleic acid does not give the reaction. The application of the Feulgen reaction to histochemical studies on animal tissues was elaborated by Cowdry (1928) and Verne (1928). Milovidov (1938) published a complete bibliography of the 450 papers dealing with the Feulgen reaction up to 1938. Since then Whitaker (1938) described a technique for plant tissues, Stowell (1945a) studied the Feulgen reaction for the photometric measurement of desoxyribonucleic acid (page 126), and Oster and associates (1942,1944) employed the histochemical approach to study tissue aldehydes in sections of fresh frozen material. An improved preparation of the Feulgen reagent was reported by Coleman (1938). Rafalko (1946) claimed that small and diffuse chromatin elements could be detected with greater delicacy when the reagent was made by decolorizing a 0.5% solution of the dye by bubbling sulfur dioxide through it.

The specificity of the Feulgen reaction for aldehydes has been brought up repeatedly. It has been variously claimed that oleic and cinnamic acids give a positive reaction, and that ketosteroids cannot be differentiated from aldehydes by the reaction. Oster and Oster (1946) have examined the question of specificity and have found that the "true" reaction is indeed specific for aldehydes, other carbonyl compounds giving a "pseudo" reaction in certain instances. The differentiation between the "true" and "pseudo" reactions may be made according to Oster and Mulinos (1944) on the basis that the purple color developed in the former can be decolorized with dilute sodium hydroxide and restored to its original intensity with hydrochloric acid, while the reddish color of the latter cannot be restored by acid after the decolorization.

A means for the microscopic demonstration of ribonucleic acid was developed by Dubos (1937) and Brachet (1940), who employed ribonuclease to break down the compound and thus destroy its basophilic staining properties. The crystalline ribonuclease prepared by Kunitz (1940) provided a more satisfactory reagent for carrying out the procedure. Opie and Lavin (1946) demonstrated that ribonucleic acid can be protected against ribonuclease by precipitation of the acid with lanthanum acetate. The basophilia of the precipitate was retained even after treatment with ribonuclease.

The danger of an uncritical acceptance of the localizations obtained by the Feulgen reaction has been emphasized by Danielli (1946a). He pointed out that it remains to be proved whether the experimental treatment of the nucleic acid has rendered it diffusible enough for this factor to become significant in the interpretation. In addition, he stressed the point that the use of an enzyme to digest away a particular substance is open to some question with reference to the specificity of the enzyme and the degree to which a clear-cut removal of the substrate is possible. On the other hand, Stowell (1946) reviewed the evidence for and against the specificity of the Feulgen technique for thymonucleic acid, and he concluded that with the proper precautions it is one of the most specific histochemical reactions. This does not mean that Stowell considers the technique beyond all criticism. No doubt he would agree with Danielli that the interpretation of the results should be tempered with a healthy awareness of the limitations involved, particularly the diffusibility factor.*

Turchini and co-workers (1943, 1944, 1945) reported the use of 9-phenyl (or methyl)-2,6,7-trihydroxy-3-fluorone for the differential staining of ribo- and desoxyribonucleic acids, the former giving rise to a yellow-pink color, and the latter to a blue-violet. It is necessary to hydrolyze the nucleic acid, as it is the pentose, thus liberated, which yields the color. The hexoses formed by the hydrolysis of tannins produce an orange-yellow color in the staining reaction when it is applied to plant tissues (Turchini and Gosselin de Beaumont, 1945).

* Other publications which have appeared subsequent to this writing are given in the Bibliography Appendix, Refs. 15, 18 and 31.

The use of ultraviolet absorption for the localization of nucleic acids is discussed on page 113.

Coleman Preparation of Feulgen Reagent

Dissolve 1 g. basic fuchsin in 200 ml. boiling water; filter, cool, and add 2 g. potassium metabisulfite ($K_2S_2O_5$) and 10 ml. 1 *N* hydrochloric acid. Let bleach for 24 hr., and then add 0.5 g. activated carbon (Norit), shake for about 1 min., and filter through coarse paper. The filtrate should be colorless.

Whitaker Feulgen Technique for Plant Tissues

SPECIAL REAGENTS

Modified Brenda Fixative. Combine 30 ml. 1% chromic acid with 10 cc. 2% osmic acid.

1 N Hydrochloric Acid.

Feulgen Reagent. See above.

45% Acetic Acid.

PROCEDURE

1. Fix tissue in the modified Brenda fluid for a period depending on the specimen, *e.g.*, 15–20 min. for root tips, 30–45 min. for whole anthers.
2. Hydrolyze in 1 *N* hydrochloric acid at 50–60° for the same time used in fixation.
3. Place in stain for 15–20 min. and then transfer to 45% acetic acid for 10–15 min. or longer.
4. Put specimen in a drop of 45% acetic acid on a glass slide and perform any dissections at this stage.
5. Place cover slip over the material and heat the slide nearly to boiling at least three times. Apply pressure to cover slip with each heating to make the tissue adhere to the slide.
6. Float off the cover slip in a mixture of equal vol. absolute alcohol and glacial acetic acid.
7. Transfer to 95% alcohol for at least 15 min. and mount in euperal. The mounting must be done in low humidity and care must be taken to avoid breathing on the slide since moisture results in cloudiness. The mounted material keeps well permanently.

Result. A positive reaction is indicated by a purple color.

Cowdry Modification of Feulgen Reaction for Paraffin Sections of Animal Tissues

SPECIAL REAGENTS

Sublimate-Alcohol Fixative. Combine equal vol. saturated mercuric chloride soln. and absolute alcohol.

1 N Hydrochloric Acid.

Feulgen Reagent. See page 67.

Sodium Bisulfite Solution. Add 30 ml. 1 *M* sodium bisulfite soln. to 600 ml. tap water.

PROCEDURE

1. Prepare paraffin sections of tissue fixed in the sublimate-alcohol fluid.
2. Pass through graded alcohols to water and place in the hydrochloric acid for 1 min.
3. Place in another portion of the acid at 60° for 4 min.
4. Treat with the Feulgen reagent for about 1.5 hr. The time may have to be varied to suit the particular sections used.
5. Pass through three separate portions of the sodium bisulfite soln. leaving in each for 1.5 min. and agitating frequently.
6. Wash for 5 min. in tap water.
7. Dehydrate, clear, and mount in balsam.

Oster Modification of Feulgen Reaction for Fresh-Frozen Sections of Animal Tissues

SPECIAL REAGENTS

1% Mercuric Chloride.

Feulgen Reagent. See page 67.

0.01 N Hydrochloric Acid Containing 1% Sodium Bisulfite.

PROCEDURE

1. Cut 50 μ sections of fresh tissue on a freezing microtome. (The sectioning should be carried out within 2–3 hr. after the death of the animal and removal of the tissue. Until ready for use, keep the tissue before cutting, and the sections after cutting, in physiological salt solution.)
2. Place the sections in 1% mercuric chloride for 5 min. in order to liberate free aldehyde from "plasmalogen." Wash with water.

3. Transfer to the Feulgen reagent for 15 min. and hold the stained sections in the hydrochloric acid-sodium bisulfite solution.

4. Examine sections immediately after washing in distilled water. The stain will last for a few days if the sections are kept in sulfurous acid solution.

Method of Turchini *et al.* for Nucleic Acids

SPECIAL REAGENTS

Nucleic Acid Reagent. Dissolve 80 mg. of 9-phenyl (or methyl)-2,6,7-trihydroxy-3-fluorone in 100 ml. 95% alcohol containing 15 drops conc. sulfuric acid.

1 N Hydrochloric Acid. (Or 25% conc. hydrochloric acid in 90% alcohol.)

1% Sodium Carbonate.

PROCEDURE

1. Fix the tissue (either plant or animal) in Bouin fluid.
2. Prepare paraffin sections in the usual manner.
3. If the methyltrihydroxyfluorone reagent is used: Hydrolyze the deparaffinized sections in 1 *N* hydrochloric acid at 60° for 5 min. wash with water, then alcohol, and treat for 5–10 min. with the reagent. Wash with several drops of 90% alcohol, then with 1% sodium carbonate, rinse with water, and finally mount in balsam.

3a. With the phenyltrihydroxyfluorone reagent: Use the same procedure as in step 3 but carry out the hydrolysis in the cold in alcoholic 25% hydrochloric acid for 3–5 min.

WATER-INSOLUBLE CARBONYL COMPOUNDS

While the fuchsin-sulfurous acid test can be used for the localization of aldehydes in tissue, other histochemical tests employed by Bennett (1939, 1940) will react with either aldehydes or ketones. Bennett concluded that his tests for the carbonyl group were indicative of ketosteroids in the outer layer of the fascicular region of the adrenal cortex. These carbonyl reactions can only indicate lipid aldehyde or ketone and are in no way specific for ketosteroids as Gomori (1942) pointed out; however, if other supporting evidence is at hand, it may be reasonable to ascribe a positive reaction to the ketosteroids present in a particular tissue. Subsequent work of Albert and Leblond (1946) indicated that "plasmalogen" rather than ketosteroids is revealed by the phenylhydrazine reaction.

Bennett (1940) first removed ascorbic acid from the tissue to prevent its interference with the tests. Albert and Leblond (1946) substituted 2,4-dinitrophenylhydrazine for the phenylhydrazine of Bennett. This enabled a more intense staining in thinner sections.

Bennett Use of Phenylhydrazine Reaction for Water-Insoluble Aldehydes and Ketones

SPECIAL REAGENTS

M/10 Acetate Buffer, pH 6.0 to 6.5.

1% Iodine in Alcohol.

1% Sodium Thiosulfate Solution.

1% Buffered Phenylhydrazine. Prepare just before use by mixing equal vol. of 2% phenylhydrazine hydrochloride and the acetate buffer. Gently bubble carbon dioxide through the solution for 15 min. to remove oxygen.

Control Reagent. Same as the 1% buffered phenylhydrazine without the phenylhydrazine.

PROCEDURE

1. Transfer frozen sections of fresh tissue from the microtome directly into acetate buffer. If fixed tissue is employed, transfer to water.
2. Add the iodine solution dropwise until a faint straw color persists, and let stand 15 min.
3. Add sodium thiosulfate solution dropwise until the color is discharged and a little more has been added; let stand 5 min.
4. Wash the sections several times in distilled water.
5. Place the sections in glass-stoppered bottles containing buffered phenylhydrazine solution. Fill the bottles to the top so that no air bubbles are present under the stopper.
6. Run control sections as in previous steps, only use the control reagent in place of phenylhydrazine.
7. After standing several hr. or overnight, wash all sections with distilled water a few times.
8. Mount in glycerol or glycerol-gelatin and examine by means of incident illumination from above.

Result. A yellow color appears in areas giving the positive test. It is essential that care be taken in conducting this test since the appearance of a yellow deposit on the walls of the bottle or on top of the liquid indicates decomposition of the reagent, and when this

occurs the yellow color in the sections cannot be relied upon to be specific for the groups tested.

Albert and Leblond Use of 2,4-Dinitrophenylhydrazine Reaction for Water-Insoluble Aldehydes and Ketones

SPECIAL REAGENTS

2,4-Dinitrophenylhydrazine Reagent. To a saturated soln. of 2,4-dinitrophenylhydrazine (No. 1866 Eastman Kodak Co.) in 30% alcohol, add sufficient 0.2 *N* sodium acetate to bring the *p*H to neutrality.

PROCEDURE

1. Fix tissue for 48 hr. in formalin (neutralized with magnesium carbonate) and wash in running water for 24 hr.
2. Prepare frozen sections 10–15 μ and place in 17% alcohol for 4 hr.
3. Place sections in the 2,4-dinitrophenylhydrazine reagent overnight and wash in 17% alcohol for 20 min.
4. Carry to distilled water and mount in glycerol gelatin.

Result. A positive reaction is shown by a yellow color.

Bennett Use of Semicarbazide Reaction for Water-Insoluble Aldehydes and Ketones

SPECIAL REAGENTS

Acetate Buffer, Iodine, and Thiosulfate Solutions. Same as in the preceding phenylhydrazine test.

Semicarbazide Reagent. Grind 10 g. semicarbazide hydrochloride with 15 g. crystalline sodium acetate, take up the mixture in 100 ml. absolute methanol, and filter.

Control Reagent. Same as the semicarbazide reagent without the semicarbazide.

PROCEDURE

1.–4. Follow the first four steps in the procedure for the phenylhydrazine test.

5. Place sections in the semicarbazide reagent.
6. Place control sections in the control reagent.
7. After overnight standing, wash all sections several times with distilled water.
8. Examine at once with incident light from above.

Result. A yellowish deposit of semicarbazones appears in the areas where the aldehydes and ketones are present.

PURINES

Tests that have been found to give positive results with all of the purines have the unhappy characteristic of being highly unspecific. Thus the reduction of silver salts is a reaction much too unspecific to merit consideration; Saint-Hilaire's method involving precipitation of insoluble copper salts of purines, and the transformation of the copper into its red ferrocyanide is a reaction also given by protamines, histones, and other protein products (Lison 1936, pages 183–186).

The murexide test, which is positive with uric acid, xanthine and its methyl derivatives, and guanine, is not given by adenine or hypoxanthine (Lison 1936, pages 186–187). This reaction has the disadvantage of being too drastic to permit its use for fine structures and its disintegrating effect on tissue sections presents technical difficulties. However, it may prove useful in some cases and for this reason it will be described. Since it is no different from the xanthoproteic reaction, a yellow-orange color would be indicative of proteins, but it should be kept in mind that the xanthoproteic test is not specific for proteins since other compounds, such as alkaloids, benzene derivatives, etc., can also be nitrated in this manner to yield products having the same color.

Cowdry (1943, page 196) suggested that the modification of the Courmont-André method by Hollande (1931) be used. It enables a more reliable localization of urates in tissue.

Murexide Test for Certain Purines

SPECIAL REAGENTS

Concentrated Nitric Acid.
Concentrated Ammonium Hydroxide.

PROCEDURE

1. Prepare sections by any of the usual methods.
2. Place a drop of nitric acid on a section and warm gently for 30 sec.

3. Drain off the acid by means of blotting paper and add a drop of water, which is also removed in the same way.

4. Expose the section to ammonia vapors.

Result. A purple-violet color is a positive test for uric acid, guanine, and xanthine and its methyl derivatives. A yellow-orange color is usually indicative of protein material. The effect of diffusibility should be considered in the interpretation of localizations.

Hollande Modification of Courmont-André Method for Uric Acid and Urates

SPECIAL REAGENTS

Silver Nitrate-Neutral Formalin Fixative. Equal vol. of 1% silver nitrate and 4.4% formalin (neutralized with calcium carbonate) mixed just before using.

0.5% Phosphomolybdic Acid.

PROCEDURE

1. Fix tissue in silver-formalin mixture for 12–24 hr. in the dark.
2. Wash for 24 hr. in several changes of distilled water.
3. Prepare paraffin sections.
4. Stain sections with hemalum for 10 min. and wash in running tap water for 30–60 min.
5. Place in 1% aqueous orange G or eosin 30–60 min. and wash rapidly in distilled water.
6. Treat with the phosphomolybdic acid soln. and wash in distilled water.
7. Stain with 0.12% aqueous light green for 1–10 min.
8. Differentiate quickly in 95% alcohol, dehydrate in isoamyl alcohol, clear in xylol, and mount in balsam.

Result. Urates will appear black, chromatin blue, protoplasmic inclusions red to orange, and collagenic fibers green.

INDOLE AND RELATED COMPOUNDS

Lison (1936, pages 160–162) lists five reactions for the histochemical detection of compounds containing the indole structure. All of the tests leave much to be desired; their specificity is rather

poor. The Ehrlich reaction employing *p*-dimethylaminobenzaldehyde will give a violet color with phenols, aryl amines, and heterocyclic compounds. Diazotization may indicate the same classes of substances. The indophenin reaction utilizing isatin and sulfuric acid gives a reddish-violet color in the presence of five-membered heterocyclic compounds including indole. The nitrosamino reaction of Lison converts the imino group in pyrrole or indole to a nitrosamine by means of nitrous acid, and the nitrosamine is then made to produce a green color through the Liebermann reagent (5% phenol and concentrated sulfuric acid). This test is given by imino groups, phenols, and primary aryl amines. The nitro reaction enables differentiation between pyrroles and indoles; the sections are treated with a mixture of equal parts of sulfuric and nitric acids, and benzene ring compounds including indoles develop a canary yellow color while pyrroles are not colored.

PHENOLS

Four main staining reactions have been employed for the detection of phenols in tissue preparations. The azo reaction is based on diazotization to form colored compounds; the indo reaction depends on the formation of a green or blue indamine when an aromatic para diamine is oxidized in the presence of tissue phenol; the "argentaffin" reaction makes use of the reduction of ammoniacal silver hydroxide and applies to ortho and para polyphenols, polyamines, and aminophenols; and the "chromaffin" reaction, which is used particularly to indicate adrenaline, gives rise to a brown color when tissue is fixed with dichromate salts. A discussion of these tests was given by Lison (1936, page 139–160). The argentaffin test is quite unspecific since many reducing substances can likewise give a positive reaction. The chromaffin test is not entirely specific for adrenaline, but has proved useful for the histochemical localization of this biologically important substance.

Lison Modification of Chromaffin Reaction

SPECIAL REAGENTS

Formol-Müller Fixative or 5% potassium iodate in 10% formalin.
3% Potassium Dichromate or Potassium Iodate.

PROCEDURE

1. Fix tissue in one of the solns. indicated. The iodate gives a less intense reaction but is less prone to pseudoreactions.
2. Prepare sections and treat them with the 3% reagent for a few hours.

Result. A brownish color indicates a positive reaction.

UREA

Two methods have been proposed for the localization of urea in tissue sections. The Leschke procedure is based on fixation of the tissue in a half-saturated mercuric nitrate solution in 1% nitric acid and subsequent treatment of the sections with a saturated hydrogen sulfide solution. The mercury urea compound is converted to black mercuric sulfide, which is easily visualized. The xanthydrol method depends on fixation of the tissue in a solution of xanthydrol in acetic acid in order to precipitate dixanthylurea which can be recognized in sections by its double refraction when examined under a polarizing microscope. Lison (1936, pages 165–170) critically discussed these methods. As he pointed out, the usefulness of the mercury reaction is entirely vitiated by its extreme lack of specificity, far too many tissue constituents being capable of precipitation by mercury salts. The xanthydrol reaction is chemically specific, but its serious fault lies in a combination of unfortunate factors including the great diffusibility of urea, the poor penetrability of xanthydrol, and the slowness of the reaction between the two. The result is that the position of the crystals formed bears little or no relation to the regions originally containing urea. A suitable method for the histological localization of urea is not available at present.

SULFONAMIDES

MacKee *et al.* (1943) described a test for sulfonamides in frozen tissue sections depending on the formation of a yellow to orange precipitate of the *p*-dimethylaminobenzylidene derivative when sulfonamides react with *p*-dimethylaminobenzaldehyde. It should be borne in mind that procaine, phenacetin, acetanilid, and aromatic amino compounds in general will also give the reaction.

Another method was published by Hackmann (1942), who employed the freezing-drying technique for the fixation of the tissue, prior to the preparation of paraffin sections. Colored sulfonamides were observed directly in the sections, and colorless ones were visualized by forming a red azo dye in the following manner: The sections were exposed to nitrous acid vapor for 30 sec. by placing the slide over a measuring cylinder 20 cm. high containing several milliliters of 0.1 *N* hydrochloric acid and a few milligrams of sodium nitrite. The diazotized sulfonamide was coupled with α-naphthylamine by immersing the slide in a 5% solution of the amine in xylol.

The detection of sulfonamides by fluorescence microscopy is discussed on page 108.

Method of MacKee *et al.* for Sulfonamides

SPECIAL REAGENTS

Sulfa Reagent. Dissolve 1 g. pure *p*-dimethylaminobenzaldehyde in a soln. of 95 ml. absolute alcohol and 5 ml. conc. hydrochloric acid. Store in a glass-stoppered amber bottle, and do not use after 2–3 weeks or when the soln. becomes yellow.

5% Concentrated Hydrochloric Acid in Absolute Alcohol.

PROCEDURE

1. Fix the tissue for 2–24 hr. with formaldehyde gas by covering the bottom of a beaker with paraformaldehyde and the top with a piece of gauze, placing the tissue on the gauze, setting the whole in a glass jar whose floor has also been covered with paraformaldehyde, and closing the jar tightly with a glass lid.
2. Cut frozen sections of the fixed tissue 10–20 μ thick and place directly on glass slides.
3. Cover each section with a drop or two of the sulfa reagent, and after 3–5 min. add a drop or two of the alcoholic hydrochloric acid soln.
4. Dry quickly without heat by absorbing excess fluid on filter paper and holding in a current of air.
5. Cover at once with a drop of damar resin in xylol (10 g. resin dissolved in 10 g. xylol) and fit cover slip, taking care to remove air bubbles. Seal edges with melted paraffin.

6. Run controls by repeating the above steps but omitting the treatment with the sulfa reagent, or repeat the complete procedure on a portion of the same kind of tissue known to be free of sulfonamides.

Result. Sulfonamides are indicated by the presence of a precipitate that ranges in color from lemon-yellow to orange. However, the color fades rapidly, particularly in the presence of air, making it necessary to examine the sections as early as possible. Colored photomicrographs should be taken not later than 3–4 hr. after the reaction has occurred.

D. ENZYMES

UREASE

Sen (1930) elaborated a method for the localization of urease in tissue sections which he employed for a study on the jack bean. The carbonic acid formed on decomposition of urea is precipitated as calcium carbonate, which may be visualized by conversion to silver carbonate and reduction of the latter to a black deposit of metallic silver; or the carbonic acid may be converted to cobalt carbonate and the latter changed to a brown or black precipitate of cobalt sulfide. The latter method is to be preferred. This principle was later employed by Gomori for the localization of the phosphoric acid liberated by phosphatases, pages 78 and 80. However, Sen digested the tissue in the substrate medium before paraffin infiltration and sectioning, and only treated the deparaffinized sections with sulfide to convert the colorless cobalt salt to the black sulfide. This procedure has many disadvantages; the schedule of Gomori, in which the sections are prepared prior to digestion, should be used instead, if the enzyme can stand the dehydration, paraffin embedding, and deparaffinization.

For jack bean tissue, Sen employed a preliminary treatment for 1 hr. with 1% cobalt nitrate in 80% alcohol followed by a 48–60 hr. digestion with a substrate medium consisting of 0.5% urea and 0.5% cobalt nitrate in 80% alcohol. The cobalt carbonate was converted to sulfide by the action of either dilute sodium sulfide or a saturated solution of hydrogen sulfide. For animal tissues, Sen used cobalt-urea solns. in graded alcohols from 60 to 80%.

ALKALINE PHOSPHATASE*

The same staining technique for the visualization of alkaline phosphatase activity was developed independently and simultaneously, oddly enough, by Gomori (1939) in Chicago and Takamatsu (1939) in Japan. Their method was based on the finding that, when sections of tissue were placed in an alkaline medium containing sodium glycerophosphate, the sites of the enzymatic liberation of phosphate could be determined if calcium ions were present to precipitate the phosphate as it was formed. The deposit of calcium phosphate then could be converted to a more easily visualized black precipitate of cobalt sulfide or metallic silver. Gomori (1939), Hepler *et al.* (1940), Takamatsu (1939), and Kabat and Furth (1941) have employed the von Kóssa silver stain; but, as Bourne (1943) has indicated, it is probably inferior to the cobalt stain used extensively in the latter work of Gomori (1941a, 1943).

The specificity of the stain for phosphatase has been demonstrated by Gomori (1939, 1941a) and Kabat and Furth (1941), and in a critical study later Danielli (1946b) claimed reliability for the localizations obtained. Preformed insoluble calcium salts will give a positive test and therefore these should either be removed by treating the sections, before incubation with substrate, with citrate buffer of *p*H 4.5 to 5.0 for 15 min. (Gomori, 1946c), or control sections stained to demonstrate the preformed salts should be compared to the sections treated to visualize the enzyme reaction. Of course the former is preferable.

Tissues too hard to be sectioned without decalcification present a particular problem since phosphatase is destroyed by the usual processes of decalcification. Kabat and Furth (1941) circumvented this difficulty to some degree by the use of 10% diammonium citrate, which they found could effect certain decalcifications without damaging phosphatase. Bourne (1943) has proposed that small pieces of bone tissue be fixed in 80% alcohol, treated with the substrate medium and then with cobalt solution and sulfide to convert the calcium phosphate precipitate to one of cobalt sulfide, and finally subjected to decalcification with trichloroacetic acid. Cobalt sulfide is insoluble in trichloroacetic acid and hence the decalcification can be performed as a final step. Controls can be made by following the

* See Bibliography Appendix, Ref. 7.

same procedure but omitting the treatment with substrate. Another procedure for use with bone has been given by Bourne (1943) involving the addition of 0.01% sodium alizarin sulfonate to Gomori's substrate medium. The calcium phosphate produced is automatically stained red by the alizarin dye.

The use of magnesium ions to activate the phosphatase was introduced by Kabat and Furth (1941) and is employed in the revised method of Gomori (1946c).

It is of interest to call attention to the different approach to the staining technique for alkaline phosphatase that was brought forward by Menten, Junge, and Green (1944). These investigators employed a reaction of the organic, rather than the phosphate, moiety of the substrate to precipitate a reddish-purple dye at loci of phosphatase action. Employing calcium β-naphthol phosphate as the substrate, β-naphthol liberated by the enzyme was made to react at once with diazotized α-naphthylamine present in the substrate solution. While this procedure can undoubtedly be applied in many instances, it would appear to offer no advantage over the Gomori method, and, as Menten *et al.* readily admit, in its present form the test is intricate and probably not well suited to routine use. Nevertheless, Yin (1945) employed this method for plant tissues in order to avoid interference by preformed phosphates. Since the preformed phosphates can be removed with citrate buffer (page 78) it would appear that interference from this source need not be made a determining factor in the choice of a method.

Gomori Revised Method for Alkaline Phosphatase

SPECIAL REAGENTS

5% Acetylcellulose (Eastman's No. 4644) in acetone. Optional.

1–2% Cobalt Acetate, Chloride, or Nitrate.

Ammonium Sulfide Solution. A few drops of yellow ammonium sulfide soln. to a Coplin jar of distilled water.

Substrate Medium, pH 9.4. (Will keep in refrigerator for months.) Combine 25 ml. 2% sodium glycerophosphate, 25 ml. 2% sodium barbital, 50 ml. distilled water, 5 ml. 2% calcium chloride, 2 ml. 2% magnesium sulfate, and a few drops of chloroform.

PROCEDURE

1. Place slices of fresh tissue, under 2 mm. thick, in chilled abso-

lute acetone and fix for 12–24 hr. in a refrigerator. Dehydrate at room temperature in two changes of absolute acetone for 6–12 hr. each time.

2. *Optional step.* To strengthen sections which tend to break up when floated on the lukewarm water after sectioning, impregnate the tissue with the acetone-acetylcellulose soln. 24 hr.

3. Drain off the fluid rapidly and place in two changes of benzol for 30 min. each.

4. Embed in paraffin not over 56° up to 2 hr. To hasten the process use 3 changes of paraffin, each for 20 min., and carry out the second change *in vacuo* in a wide-mouth bottle with a one-hole rubber stopper fitted with a glass tube. Connect the glass tube by rubber tubing, passed through an air hole in the paraffin oven, to a water aspirator via a safety bottle.

5. Cut sections 4–8 μ thick, float them on lukewarm water (30–35°), and mount on slides.

6. Let slides dry, place in the paraffin oven for 10 min. to melt the paraffin, and run through xylol and alcohols to distilled water. Remove preformed mineral deposits as described on page 78.

7. Incubate the sections for 1–2 hr. at 37° in the substrate medium.

8. Rinse with water, immerse in the cobalt soln. for 5 min., and rinse well with several changes of distilled water.

9. Place in the diluted ammonium sulfide soln. for 1–2 min.

10. Wash well in water, counterstain if desired, dehydrate, and mount.

Result. Sites of the phosphatase activity appear brown or black.

ACID PHOSPHATASE*

The staining method for alkaline phosphatase cannot be used for acid phosphatase since calcium phosphate is soluble at a *p*H around 5, which is optimum for the action of the latter enzyme. Hence, Gomori (1941b) employed lead ions in the substrate medium at *p*H 4.7 so that insoluble lead phosphate would be formed at the sites of enzymatic activity. The lead phosphate was then converted either to brown or black lead sulfide, or was stained a purplish-red with acridine red. Wolf, Kabat, and Newman (1943) introduced several

*** See Bibliography Appendix, Refs. 1 and 9.**

improvements in the procedure, and subsequently Gomori (1946c) revised his original method. His experience with the technique led Gomori (1946c) to state: "For some unknown reason, the staining for acid phosphatase sometimes turns out patchy, occasionally even negative, when it should be positive. This seems to happen especially in cases when the pieces have been exposed to the temperature of the paraffin oven for more than an hour, or when the temperature of the oven is over 56°C."

The intensification of the acid phosphatase test by manganese ions has been demonstrated by Moog (1943a), who found that a concentration of 0.01 *M* manganese sulfate gave the most satisfactory results. This investigator recommends that the activator be added to a clear portion of substrate medium just before use, and points out that the incubation period may be approximately halved when the reaction is accelerated by the manganese. Moog found that 4–5 hr. was a satisfactory incubation period for tissues of the 6-day chick embryo. No doubt a certain amount of trial and error must be applied to determine the proper incubation time for the particular tissue under investigation. In a later study Moog (1944) found that 0.01 *M* ascorbic acid activated acid phosphatase and in some respects appeared to have advantages over the action of manganese sulfate.

The application of the Gomori method to grains and sprouts was made by Glick and Fischer (1945b). The modification in technique necessitated for these tissues will be presented.

Gomori Revised Method for Acid Phosphatase in Animal Tissues

SPECIAL REAGENTS

5% Acetylcellulose (Eastman's No. 4644) in acetone. Optional.

2% Acetic Acid.

Ammonium Sulfide Solution. A few drops of yellow ammonium sulfide soln. to a Coplin jar of distilled water.

Substrate Medium, pH 5. Combine 30 ml. of 1 *M* acetate buffer (100 ml. 13.6% sodium acetate, $CH_3COONa.3H_2O$, + 50 ml. 6% acetic acid), 10 ml. 5% lead nitrate, and 60 ml. distilled water, and add slowly while stirring 30 ml. 2% sodium glycerophosphate. Shake the mixture, let stand for a few hr., and store in a refrigerator. Before use, filter a small amount and dilute it with 2–3 parts distilled water.

PROCEDURE

1–6. Same as for alkaline phosphatase (pages 79 and 80).

7. Incubate the sections for 1–24 hr. at 37° in the substrate medium.

8. Rinse well in distilled water, followed by 2% acetic acid, and then in distilled water again.

9–10. Same as for alkaline phosphatase, page 80.

Result. Sites of the phosphatase activity appear brown or black.

Glick and Fischer Adaptation to Grains and Sprouts of Gomori Method for Acid Phosphatase

SPECIAL REAGENTS

Substrate Medium. Combine the following, shake thoroughly, centrifuge, and use the clear liquid: 4 ml. 0.1 *M* acetate buffer of *p*H 5.1, 1 ml. 0.1 *M* lead nitrate, 0.6 ml. distilled water, and 0.4 ml. 3.2% sodium-α-glycerophosphate. Booth (1944) showed that the α compound is hydrolyzed more rapidly than the β by wheat phosphatase, and Gomori (1941) found that the α compound has the additional advantage that its lead salt is more soluble than that of the β at this *p*H value. Because it was easily available, the mixture, containing 52% α and 48% β of *Eastman Kodak Co.,* was used by Glick and Fischer (1945).

2% Acetic Acid Solution.

Ammonium Sulfide Solution. 1 ml. to a Coplin jar of water.

PROCEDURE

I, Preparation of paraffin sections. A, Kernel sections:

1. Soak kernels in water about 7 hr.

2. For longitudinal sections, cut off a layer from both the crease side and the opposite side of the kernel. For cross sections, cut off kernel just behind germ. This enables more efficient penetration of liquids.

3. Let kernels stand overnight in absolute alcohol. In the morning change to a mixture of 1 vol. absolute alcohol + 3 vol. *n*-butyl alcohol.

4. In the evening transfer to *n*-butyl alcohol.

5. The following morning place in xylol, and let stand until evening.

6. Transfer to a xylol-paraffin mixture containing just enough xylol to keep the paraffin in soln. at room temperature and let stand overnight. Tissuemat (*Fisher Scientific Co.*) gives better results than paraffin. In a warm room the variety melting at 60–62° gives better sections than the material having a lower melting point.

7. Place in a soln. of 1 vol. xylol + 2 vol. melted paraffin in a 60° oven for 1–2 hr.

8. Infiltrate with melted paraffin for 2 hr. in the oven, then change to fresh paraffin for 4 hr., and finally embed.

9. Cut sections 10 μ thick and mount on slides with aid of Mayer albumin. (Combine 1 vol. filtered fresh egg white with 1 vol. glycerol, and add a bit of camphor as a preservative.) Smear the liquid in a thin film on a slide and rub with the finger, cover with water, transfer section to the slide, place in oven for 5 min. at about 55° to soften the paraffin and allow wrinkles to straighten out, drain off water with a towel, and allow to dry for 2 hr. in the 55° oven. Store mounted sections in refrigerator until ready for use.

10. Remove paraffin from sections with two changes of xylol followed by two changes of absolute alcohol.

11. Dip slides into 0.5–1.0% collodion in alcohol-ether to cover section with a protective film; harden film by dipping into 80% alcohol, and wash with distilled water.

B. Rootlet and leaf section of the sprout:

1. Place in the following solns., for 1 hr. in each case, in the order given:

   a. 70% alcohol
   b. 80% alcohol
   c. 65 ml. 80% alcohol ÷ 35 ml. *n*-butyl alcohol
   d. 45 ml. 95% alcohol ÷ 55 ml. *n*-butyl alcohol
   e. 25 ml. absolute alcohol ÷ 75 ml. *n*-butyl alcohol
   f. *n*-butyl alcohol
   g. xylol

2. Follow step 6 under *A* in the preceding part, allowing the material to stand in the mixture for only ½ hr.

3. Subject the material to three changes of melted paraffin in the

60° oven during the course of 1 hr. If air bubbles are present in the leaves, apply suction to remove them.

4. Embed in paraffin colored red by stirring a few grains of Sudan IV in the molten material. In uncolored paraffin it is difficult to see the tissue in the sections.

5. Cut sections, mount on slides, remove paraffin, and protect with collodion film just as in steps 9, 10, and 11 under *A*.

II, Preparation of frozen sections. A, Kernel sections (rootlet and leaf sections of the sprout are too fragile to permit satisfactory frozen-section technique):

1. Soak kernels 4–6 hr. in water.
2. Mount in a drop of water on freezing head of microtone.
3. Cut sections 15 μ thick, keeping knife cold with Dry Ice, and transfer, with a needle cooled by Dry Ice, into 80% alcohol. (The 80% alcohol is used rather than water since, in the latter medium, the starch endosperm disintegrates, and separates from the rest of the section.)
4. Float the section onto a glass slide immediately. If wrinkled, straighten section in a drop of 70% alcohol.
5. Dehydrate by covering section with five successive drops of absolute alcohol, draining off after each drop is added.
6. Cover section with a small drop of 0.5–1.0% collodion soln. and harden film by dipping slide in 80% alcohol. Wash in distilled water.

III, Demonstration of enzyme activity:

1. Remove preformed mineral deposits by placing sections in distilled water for 24 hr. at room temperature. Citrate buffer could probably be used too (page 000).
2. Place sections in substrate medium at 37°, for the following digestion periods:

Kernel, paraffin sections—Embryo, 1 hr.; non-embryonic part 30 min.

Kernel, frozen sections—Embryo, 15–30 min.; non-embryonic part, 5–10 min.

Rootlets, paraffin sections—3 hr.

Epicotyl, paraffin sections—24 hr.

3. Wash sections with three changes of distilled water, dip into 2% acetic acid, and wash well with distilled water.

4. Place in ammonium sulfide soln. for 2–3 min.
5. Wash with several changes of distilled water, dehydrate in 95% alcohol for 2–3 min., and follow by 5 min. in absolute alcohol.
6. Clear in oil of thyme for 3–4 min., treat with three changes of xylol. (Treatment with xylol should be brief since the black precipitate indicating enzyme action is soluble to some degree in xylol.) Mount in balsam.

Result. The phosphatase activity is visualized as a brown or black precipitate.

OTHER PHOSPHATASES

Various investigators have studied the histological distribution of phosphatases capable of hydrolyzing substrates other than those commonly employed for the acid and alkaline phosphatases. This work has been accomplished by simply substituting the new substrates for the glycerophosphate usually used, and employing the standard phosphatase procedures.

Wolf, Kabat, and Newman (1943) used ribonucleic acid and glucose-1-phosphate as additional substrates in their work on acid phosphatase distributions, particularly in the human and guinea pig nervous systems. Glick and Fischer (1945b, 1946a) employed adenosine triphosphate, thiamine pyrophosphate, and glucose-1-phosphate in a study of the enzyme distributions in wheat and parts of the germinated grain. In investigations on the mouse duodenum, Dempsey and Deane (1946), and in work on the thyroids of various species, Dempsey and Singer (1946), utilized adenylic acid, ribonucleic acid, glucose-1-phosphate, fructose diphosphate, and lecithin as their additional substrates. Krugelis (1946) studied the phosphatases in the larval salivary glands of *Drosophila* and in various organs of the mouse using adenylic acid, guanylic acid, cytidylic acid, ribonucleic acid, desoxyribonucleic acid, and a depolymerized form of the latter, as substrates.

With the staining technique it is difficult at times to define the specificities of the various phosphatases which act on the different substrates. For instance, both alkaline phosphatase and adenylpyrophosphatase (adenosinetriphosphatase), which are known to be two distinct enzymes, can act on adenosine triphosphate, as

Moog and Steinbach (1946) have emphasized. Accordingly, differences in their localizations or in their properties must be exploited to enable their separate identification in tissue sections (Glick, 1946). From the work of Dempsey and Deane (1946) it would appear that several phosphatases may coexist in the same cellular location, and that their differentiation must depend on differences in *p*H optima or other properties. When glucose-1-phosphate is employed as the substrate, enzymatic liberation of phosphate might occur either by phosphatase action or by the phosphorylase action which converts the substrate to glycogen or starch; accessory evidence would be required to determine which of these two enzymes was being visualized by the staining reaction.*

While it does not offer rigorous proof, in some cases differentiation between enzymes may be based on differences in their localization as seen in stained sections. The presence in the nucleoli of the cells of the wheat epicotyl of an enzyme capable of hydrolyzing adenosine triphosphate, but not thiamine pyrophosphate (Glick and Fisher, 1946a), would suggest that these substrates are acted upon by different enzymes. Likewise, the fact that a strong enzymatic activity is found in the cytoplasm of cells in mouse tissues when ribonucleic acid is used as the substrate, while only a slight reaction is observed in the nuclei, and the reverse distribution is seen when a depolymerized form of desoxyribonucleic acid is used, indicates that separate enzymes are involved in the hydrolysis of these substrates (Krugelis, 1946). Furthermore, the approximately equal activities in both cytoplasm and nucleus toward glycerophosphate as substrate might be taken as an indication of the presence of a third enzyme in these cells, as Krugelis has pointed out. Another example is to be found in the differences in the localizations of the enzymes acting on glucose-1-phosphate and fructose diphosphate when the mucosa of the mouse duodenum is studied (Dempsey and Deane, 1946). Other cases might be cited to illustrate the same general point.

ZYMOHEXASE (ALDOLASE plus ISOMERASE)

Aldolase converts hexose diphosphate to *both* dihydroxyacetone phosphate and phosphoglyceraldehyde; isomerase catalyzes equi-

* See Bibliography Appendix, Ref. 19.

librium between the two. Together these two enzymes are referred to as zymohexase.

Allen and Bourne (1943) adapted the microscopic technique for phosphatase (page 78) to this enzyme system, whose distribution they studied in skeletal, heart, and smooth muscle tissue. By incorporating iodoacetic acid into their substrate media, they prevented further enzymatic breakdown of the triose phosphates. The distinct difference in localization of zymohexase and alkaline phosphatase precluded the possibility of confusing the two; however, the phosphatase activity could be selectively blocked by fluoride. Allen and Bourne utilized the fact that the triose phosphates formed by the zymohexase action will spontaneously liberate inorganic phosphate at room temperature in alkaline solution. The phosphate could then be precipitated, and finally visualized in the manner of Gomori (page 78). It was observed that sections which had been infiltrated with paraffin lost their enzyme activity and, accordingly, frozen sections were employed.

Allen and Bourne Method for Zymohexase

SPECIAL REAGENTS

0.1 M Sodium Iodoacetate. Neutralize 1.86 g. iodoacetic acid with 1 *N* sodium hydroxide to bromothymol blue and dilute to 100 ml.

0.1 M Sodium Fluoride.

2% Cobalt Chloride ($CoCl_2.6H_2O$).

Ammonium Sulfide Solution. Dilute 1 ml. yellow ammonium sulfide to 50 ml. with distilled water. Prepare fresh before use.

Magnesia Mixture. Dissolve 5.5 g. magnesium chloride ($MgCl_2.6H_2O$) and 7.0 g. ammonium chloride in 35 ml. 5 *N* ammonium hydroxide. Filter after 1 hr. and add 60 ml. 4 *N* ammonium hydroxide to the filtrate.

Purified Sodium Hexose Diphosphate. Formula I: mix 40 ml. 4% sodium hexose diphosphate (prepared by treating the calcium salt with sodium oxalate) and 20 ml. magnesia mixture, and filter off precipitated phosphate after 30 min. Formula II: mix 20 ml. of 4% sodium hexose diphosphate with 20 ml. of magnesia mixture and 20 ml. of distilled water; filter after 30 min. as above.

Substrate A. Combine 10 ml. of the purified salt soln. (Formula I) with 1.7 ml. of 0.1 *M* sodium iodoacetate and 5 ml. distilled water.

Substrate B. Same as *A*, but use 3.3 ml. water and 1.7 ml. of 0.1 *M* sodium fluoride in place of the 5 ml. water. (Substrate B was used to prevent hydrolysis by phosphatase through the action of the fluoride. Actually Allen and Bourne found that it was not needed in their work since no phosphatase action was observed in their particular experiments.)

Substrate C. Combine 15 ml. of the purified salt soln. (Formula II) with 2.5 ml. of the sodium iodoacetate and 7.5 ml. water.

PROCEDURE

1. Fix tissue in 80% alcohol for 24 hr.
2. Wash in water for 5–10 min.
3. Prepare frozen sections and place them in either substrate A or C for 1–2 hr. at 37°. (An extraneous dark precipitate forms on the surface of some of the sections with substrate A, but not with C, presumably because of the higher dilution of the substrate in the latter.)
4. Treat the sections with the cobalt chloride soln. for several hr. and then with the ammonium sulfide soln. for 10 min.
5. Dehydrate in alcohols, clear in xylol, and mount in balsam.
6. Prepare control sections to demonstrate preformed phosphate by omitting the treatment with substrate in step 3 and proceeding with steps 4 and 5.

Result. The formation of a brownish-black precipitate indicates zymohexase activity.

LIPASE

Gomori (1945b) adapted the principle of his method for the demonstration of phosphatases in tissue sections to the localization of lipase. The great difficulty encountered in previous attempts has been to find a substrate that is water soluble and whose acid split-product could be precipitated by some ion having no adverse effect on the enzyme. The ordinary esters of mono- and dicarboxylic acids do not meet both of these requirements. Gomori was able to circumvent the difficulty by the use of some new long-chain fatty acid esters of hexitans and hexides in which most of the hydroxyl groups are etherified. These compounds were developed by *Atlas Powder*

Co., and are known as "Tweens." Tween 40 and Tween 60, employed by Gomori, are described by *Atlas Powder Co.* as "sorbitan monopalmitate polyoxyalkylene derivative" and "sorbitan monostearate polyoxyalkylene derivative," respectively. Gomori states that these substrates were found to be hydrolyzed by pancreatic lipase at a rate about half that of olive oil. In the presence of 0.2% sodium taurocholate an intensification of the reaction in pancreatic tissue was noted, while in all other tissues, enzyme inhibition was observed.

In a later publication Gomori (1946a) reported that "Product 81" of *Onyx Oil and Chemical Co.* could also serve as a lipase substrate for histochemical purposes. This compound is a stearic acid ester of "comparatively short-chained polyglycols." The only shortcoming observed by Gomori to the use of these substrates is an occasional failure to obtain proper counterstaining with hematoxylin, especially after use of the "Tweens" and more rarely with "Product 81." The method as finally given by Gomori (1946c) will be described.

Gomori Revised Method for Lipase

SPECIAL REAGENTS

5% Acetylcellulose (Eastman's No. 4644) in acetone.

1–2% Lead Nitrate.

Ammonium Sulfide Solution. A few drops of yellow ammonium sulfide soln. to a Coplin jar of distilled water.

Substrate Medium. Stock solution I: combine 150 ml. glycerol, 50 ml. of 10% calcium chloride, 50 ml. *M*/2 maleate buffer *p*H 7 to 7.4 (dissolve 5.8 g. maleic acid in 94 ml. of 4% sodium hydroxide and 6 ml. water), and distilled water to make 1000 ml. Stock solution II: 5% Tween 40 or 60 or "Product 81." Add merthiolate to 0.02% in each stock soln. and store in refrigerator; the solns. may be used for many months. Before use, add 2 ml. stock soln. II to 50 ml. stock soln. I.

PROCEDURE

1–6. Same as for alkaline phosphatase (pages 79 and 80).

7. Incubate the sections in the substrate medium for 6–12 hr. at 37°.

8. Rinse with distilled water and transfer to the lead nitrate soln. for 10 min.

9. Rinse in repeated changes of distilled water and immerse in the diluted ammonium sulfide soln. for 1–2 min.

10. Wash well in water, counterstain lightly with hematoxylin and eosin, dehydrate in alcohols, clear in gasoline or tetrachloroethylene, and mount in Clarite dissolved in the same liquid. (Toluol or xylol causes fading of the stain.)

Result. Sites of lipase activity appear golden brown.

PEROXIDASE

Most of the various microchemical methods for the histological localization of peroxidase activity are based on the oxidation of benzidine. The methods of McJunkin (1922), designed for use with human tissues, and Armitage (1939), developed for examining blood and bone marrow smears, have been chosen for presentation here since they are the most recent and seem to be the best. Peroxidase actually occurs most abundantly in plants, but the methods appear to have been worked out for animal tissues or cells exclusively. However, there appears to be no reason why these methods cannot be adapted to plant material as well.

McJunkin Method for Peroxidase in Tissue Sections

SPECIAL REAGENTS

Benzidine Reagent. Dissolve 100 mg. benzidine in 25 ml. 80% methanol and add 2 drops 3% hydrogen peroxide. Dilute with 1–2 vol. distilled water before using. Store in the dark.

PROCEDURE

1. Place formalin-fixed tissue, in pieces 1 mm. thick, in 70% acetone for 1 hr. followed by pure acetone for 30 min., benzol for 20 min., and melted paraffin 20 min.

2. Cut sections 3.5 to 5.0 μ, fix to slides with albumin, and dry overnight at room temperature.

3. Remove paraffin by placing in benzol for 20 sec. and acetone for 10 sec.

4. Plunge in water for a few sec. and remove excess water. Apply the benzidine reagent for 5 min. and transfer to water for 5 min.

5. The sections may be stained with Harris hematoxylin for 2 min., rinsed in water 1 min., and stained with 0.1% eosin for 20 sec.

6. Dehydrate in 95% alcohol for 30 sec., and absolute alcohol for 5 sec.

7. Clear in xylol and mount in balsam.

8. Run controls in which the benzidine is omitted.

Result. Peroxidase manifests itself by an initial blue color which changes to brown. Diffusibility, particularly of the color produced, can be expected to interfere with proper localization of the enzyme.

Armitage Method for Peroxidase in Blood or Bone Marrow Smears

SPECIAL REAGENTS

Fixing Solution. 10% Formalin in 96% alcohol. Prepare the soln. just before using.

Benzidine Reagent. Dissolve 750 mg. benzidine in 500 ml. 40% alcohol, filter, add 0.7 ml. 3% hydrogen peroxide, and shake before using. If stored in the dark, the reagent will be good for months.

PROCEDURE

1. Fix the smear in the alcoholic formalin.

2. Cover the material with the benzidine reagent for about 2 min. if the smear is fresh, and up to 20 min. if it is old.

3. Wash in 40% alcohol until yellow granules appear in the leucocytes.

4. Dehydrate in absolute alcohol and dry at about 37°.

5. A counterstain of dilute Giemsa or dilute Leishman stain may be applied for 30 min. followed by washing in water, blotting, and drying.

Result. The appearance of yellow granules is a positive test for peroxidase.

DOPA OXIDASE

The enzymatic oxidation of 3,4-dihydroxyphenylalanine—or dopa—has been applied, histologically, to the identification of melanoblasts, since these appear to be the seat of the oxidase activity and the conversion of dopa to melanin results in their becoming blackened. Bloch's earlier work has been adapted by Laidlaw (1932) and Laidlaw and Blackberg (1932) to the demonstration of dopa oxidase

activity in histological preparations. Sharlit *et al.* (1942) have pointed out that the method for demonstrating dopa oxidase may of itself, in the absence of substrate, cause an increase in melanin. This makes it necessary to run suitable control experiments. The reaction may be hastened by employing a buffer of a little higher *p*H, or retarded by shifting toward the acid side.

Laidlaw Method for Dopa Oxidase

SPECIAL REAGENTS

Dopa Stock Solution. Dissolve 0.3 g. dopa (*Hoffmann-La Roche,* labeled "for Bloch's dopa reaction") in 300 ml. cold distilled water. Store in a refrigerator and discard when a distinct red color has developed.

Buffered Dopa Solution (*p*H 7.4). Add 2 ml. potassium dihydrogen phosphate (9 g. KH_2PO_4/l.) and 6 ml. disodium hydrogen phosphate (11 g. $Na_2HPO_4.2H_2O$/l.) to 25 ml. dopa stock soln. Filter through fine paper.

Buffer Solution for Control Experiment. Replace the 25 ml. dopa soln. with an equal vol. distilled water in the buffered dopa soln. above.

PROCEDURE

1. Prepare frozen sections of fresh tissue. (It is stated that tissue hardened in 5% formalin for 2–3 hr. may be used.)
2. Rinse in distilled water for a few sec. and transfer at once to the buffered dopa soln. At 30–37° the soln. becomes red in about 2 hr. and sepia brown in 3–4 hr. Do not let the sections remain in the soln. once it becomes sepia colored since overstaining may result. Examine from time to time under a microscope to determine the proper intensity of staining. It is good practice to change to a fresh dopa soln. after the first 30 min.
3. Wash sections in distilled water, dehydrate, and counterstain with alcoholic cresyl violet or methyl green-pyronine.
4. Clear and mount in balsam.
5. Run controls by treating tissue as above with the substitution of buffer soln. for the buffered dopa soln. in step 2.

Result. Dopa oxidase is indicated by blackening in the sections. Leucocytes and melanoblasts appear grey or black due to their dopa oxidase content. Melanin maintains its natural yellow-brown color, and collagen appears colorless or pale grey.

AMINE OXIDASE

Oster and Schlossman (1942) developed a histochemical method for the demonstration of amine oxidase based on the detection of the aldehyde formed as the product of amine oxidation. The fuchsin-sulfurous acid reagent of Feulgen was used for the visualization of the aldehyde (page 65). Naturally occurring aldehydes and "plasmal" are prevented from interfering with the test by binding them with bisulfite prior to the application of the tyramine substrate solution. The diffusibility of the color produced subjects the localizations which may be observed to criticism.

Oster and Schlossman Method for Amine Oxidase

SPECIAL REAGENTS

2% Sodium Bisulfite Solution.

Substrate Solution. 0.5% tyramine hydrochloride in $M/15$ phosphate buffer of pH 7.2.

Control Solution. Omit the tyramine in the substrate soln.

Feulgen Reagent. See page 67.

PROCEDURE

1. Place frozen sections of fresh tissue in 2% bisulfite solution at 37° for 24 hr. Wash thoroughly and test some of the sections with the fuchsin-sulfurous acid reagent—the test should be negative (no color) indicating all free aldehyde has been bound.
2. Incubate sections in the substrate solution for 24 hr. at 37°. Run parallel controls with the control solution.
3. Immerse in fuchsin-sulfurous acid reagent.
4. Examine sections when the rapidly formed blue color seems to be maximum.

Result. Regions of enzymatic activity appear blue, offering a distinct contrast to the reddish-purple given by "plasmal" (see page 65).

CYTOCHROME OXIDASE

Tests for cytochrome oxidases have been adapted to histochemical work and a discussion of them has been given by Lison (1936, pages 269–290). The enzyme has been referred to as "nadi oxidase" and "indophenol oxidase," but Keilin and Hartree (1938) have made it clear that it should be called "cytochrome oxidase" since its catalytic effect applies to the oxidation of reduced cytochrome. In the presence of cytochrome c, cytochrome oxidase effects the oxidation of a mixture of *p*-aminodimethylaniline and α-naphthol (nadi reagent) to indophenol, or of *p*-phenylenediamine to the diimine. The diffusibility of the colored compounds produced must be considered with reference to localizations of the enzyme in tissue.

In order to check whether a nadi reaction is being given by cytochrome oxidase or some other factor, Moog (1943b) exposed fresh tissue (chick embryo) to a 0.005 *M* azide solution in acidified physiological saline (*p*H 5.8) for 3 min., and then transferred it to freshly prepared nadi reagent containing 0.005 *M* azide. Azide specifically inhibits cytochrome oxidase. As a control of the possibility that indophenol blue might be reduced to the leuco form as fast as formed, Moog also placed the tissue in 0.003 *M* phenylurethan in saline for 3 min. to saturate the reducing systems, and then transferred to the nadi reagent containing 0.003 *M* phenylurethan. The reagent used by Moog was prepared by combining, just before use, equal parts of 0.01 *M* *p*-aminodimethylaniline in 1% sodium chloride, 0.01 *M* α-naphthol in 1% sodium chloride, and 0.066 *M* phosphate buffer. The reaction was carried out at 38° for the interval required to attain a standard coloration (5–14 min.). Under the conditions employed, identical results were obtained at *p*H 5.8 and 7.2.

Graff Method for Cytochrome Oxidase in Fixed Tissue ("M. Nadi Oxidase")

SPECIAL REAGENTS

α-Naphthol Solution. Boil 1 g. α-naphthol in 100 ml. distilled water and add 25% potassium hydroxide dropwise until the melted α-naphthol is dissolved. Store in the dark; keeps for at least 1 month.

1% p-Aminodimethylaniline or Its Hydrochloride. Boil to dissolve solid in the water. Store in the dark; may be used for 2–3 weeks.

The hydrochloride is favored because it is more stable.

Nadi Reagent. Prepare just before using by combining equal vol. of the α-naphthol and *p*-aminodimethylaniline solns. and filtering.

Strong Ammonium Molybdate Solution or Dilute Lugol Solution. Concentration not stated.

Dilute Lithium Carbonate Solution. Concentration not stated.

PROCEDURE

1. Fix tissue for two hr. in formalin vapor or in a mixture of 10 ml. formalin and 40 ml. 96% alcohol.
2. Prepare frozen sections and place them on slides which are then laid in a thin layer of nadi reagent in a petri dish. Oxygenation of the fluid is effected by careful agitation. After 1–5 min., rinse in water and examine.
3. Make the color more permanent by treating for 2–3 min. with dilute Lugol soln. The Lugol soln. converts the blue granules to brown. Washing sections in dilute lithium carbonate restores the blue. Strong ammonium molybdate soln. has been used instead of Lugol soln.
4. Counterstain with Bismark brown, safranine or alum carmine and mount in glycerin or glycerin jelly.

Result. Cytochrome oxidase is supposed to be indicated by the blue coloration.

Graff Method for Cytochrome Oxidase in Fresh Tissue ("G. Nadi Oxidase")

The *p*H of the nadi reagent must be adapted to the requirements of the particular material under investigation. Lison (1936, page 274) states that the *p*H range 7.8 to 8.2 is most suitable for animal tissues and 3.4 to 5.9 for plant material.

SPECIAL REAGENTS

α-Naphthol Solution. Prepare a 10% alcoholic soln. and just before use dilute 100 times with distilled water.

0.12% p-Aminodimethylaniline Hydrochloride. Store in the dark.

Nadi Reagent. Prepare just before using by combining equal vol. of the diluted α-naphthol soln. and the *p*-aminodimethylaniline soln.

Buffered Nadi Reagent. Mix the reagent with the suitable buffer

(acetate, phosphate, glycine, and carbonate buffers have been employed) in the respective proportion of 50:10 or 5:20.

5% Potassium Acetate Solution.

PROCEDURE

1. Prepare frozen sections directly from very fresh tissue.
2. Repeat step 2 in the preceding cytochrome oxidase method, but wash sections with physiological saline instead of water.
3. Nuclei may be stained with lithium carmine.
4. Examine under a microscope with the section covered with potassium acetate soln. Permanent preparations cannot be made.

Result. The blue or blue-violet color is also produced in this case.

Loele Method for "α-Naphthol Oxidase"

SPECIAL REAGENTS

Naphthol Reagent. Add 10% potassium hydroxide dropwise to a little α-naphthol in a test tube until the α-naphthol is dissolved. Add 200 ml. distilled water, and after 24 hr. the reagent may be used for about 3 weeks.

PROCEDURE

1. Prepare frozen sections of formalin-fixed tissue.
2. Treat sections with the α-naphthol reagent and observe effect under the microscope within a few minutes.

Result. Violet or black granules which soon disappear are supposed to be indicative of α-naphthol oxidase.

SUCCINIC DEHYDROGENASE

Semenoff (1935) gave a method for the localization of succinic dehydrogenase in tissue sections which depends on the reduction of methylene blue. The diffusibility of the dye should obviate the possibility of good localizations by this method.

Semenoff Method for Succinic Dehydrogenase

SPECIAL REAGENTS

Substrate Medium. To 2 ml. 0.05% methylene blue add 2 ml.

10% sodium succinate and make up to 10 ml. with *M*/15 phosphate buffer, *p*H 7.6 to 8.0.

Control Medium. Omit the succinate in the substrate medium.

PROCEDURE

1. Prepare fresh frozen sections.
2. Treat sections 10–15 min. with the substrate medium under a cover slip, taking care to avoid air bubbles. Seal edges of cover slip with paraffin to exclude air.
3. Observe under microscope and compare with section in control medium treated in the same fashion.

Result. Fading of dye characterizes the enzyme activity.

III. PHYSICAL METHODS

A. FLUORESCENCE MICROSCOPY

The detection and localization in tissues and cells of certain substances by virtue of their flourescent properties when subjected to ultraviolet irradiation is finding increasing application. Substances investigated in this manner include naturally occurring compounds, such as vitamin A, and others introduced into organisms for experimental purposes, such as 20-methylcholanthrene. The fluorescence exhibited in tissues or cells may be "primary," *i.e.*, produced directly by certain compounds, or "secondary," *i.e.*, resulting from treatment with so-called "fluorochromes," fluorescent substances taken up selectively by particular cellular structures having no fluorescence of their own. For the most part, the use of fluorochromes is limited to purely morphological studies without regard to chemical nature and hence need not be discussed here, except as applied to lipids (page 105). However, for those who may be interested, lists of fluorochromes and their properties may be found in Haitinger (1938), Jenkins (1937), and Metcalf and Patton (1944). General discussions of fluorescence microscopy are to be found in Sutro (1936), Jenkins (1937), Ellinger (1940), Simpson (in Cowdry, 1943, pages 76–78), and Metcalf and Patton (1944). The books of Haitinger (1938), Radley and Grant (1939), and Pringsheim and Vogel (1946) are useful for reference.

1. Apparatus

The set-up of the fluorescence microscope is shown in Figure 3. Ultraviolet radiation having a high intensity in the range 300–400 mμ is produced by means of a carbon arc or one of the mercury vapor

lamps (*A*) such as those manufactured by *Hanovia Chemical Co.* or the H3 or H4 lamps of *General Electric Co.* or *Westinghouse Electric Co.*

The ultraviolet radiation is passed through a filter (*F*) to screen out visible light. A variety of filters may be used for this purpose. The Corning color glass filter No. 584 (new No. 5840) may be used in combination with a 5–10% copper sulfate solution, to which a drop or two of sulfuric acid has been added, contained in a quartz or other cell transmitting ultraviolet rays. The copper sulfate solution may be replaced by a Corning glass filter No. 428 (4308), but the latter does not remove the heat rays as well as does the solution, and cannot be adjusted to completely absorb red light. Other filters that may be employed are a combination of the Shott glass filters UG2 and BG14, the Corex filter of nickel oxide glass, or the Uvet glass filters with a copper sulfate solution.

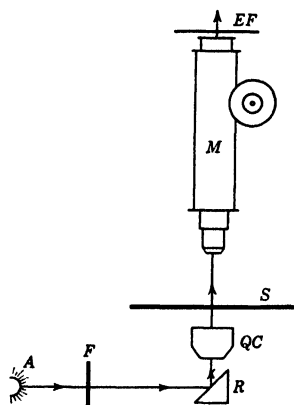



Fig. 3. Diagram of apparatus for fluorescence microscopy.

The filtered ultraviolet radiation practically free of visible light is directed into a substage condenser (*QC*), made of quartz or ultraviolet-transmitting glass, by means of a reflector (*R*) consisting of either a quartz prism, a polished mirror of aluminum-magnesium alloy, or, if the ultraviolet intensity is great, the usual plane microscope mirror. Of course, when the apparatus is aligned either vertically or horizontally on a single optical axis, the reflector is omitted. In those instances in which the fluorescence is generated by the longer wavelengths of ultraviolet radiation, as is the case for vitamin A, it is often possible to employ the ordinary substage condenser, rather than one made of quartz or special glass, since the usual grade of optical glass does not absorb much of the radiation in this range. The condenser may be eliminated entirely if radiation of lesser intensity can be used. Of the three most common forms of condensers, the aplanatic gives the best results, although the Abbe is generally quite satisfactory; achromatic condensers reduce the intensity of the radiation due to the absorption of their many lenses.

Metcalf and Patton (1944) suggest the use of a drop of water or petrolatum on the top of the condenser to serve as a connecting fluid between condenser and slide in order to obtain illumination of high intensity when objectives of twenty times magnification, or higher, are used. With low-power objectives, they point out that it is necessary to remove the top lens or lenses of the condenser so that the field can be properly illuminated. Other investigators have employed sandalwood or Shillaber oil between the slide and condenser.

The specimen is mounted on a slide (*S*) made of an ultraviolet-transparent glass such as the Corex D slide of *Corning Glass Co.* However, Metcalf and Patton (1944) have found that ordinary glass slides of 1.2 to 1.5 mm. thickness may be used when the intensity of the ultraviolet radiation is great. A nonfluorescing medium must be used for mounting the specimen; glycerol or mineral oil was recommended by Simpson (in Cowdry, 1943, pages 76–78); but Popper (1944) reported a disturbing fluorescence from glycerol (although others beside Simpson have found no difficulty with it) and the use of mineral oil is limited to substances that will not dissolve in it, *e.g.*, for vitamin A studies Popper (1944) used water as the mounting medium. In some instances, petrolatum serves as a good temporary mount, and, for permanent mounts, isobutyl methacrylate (*du Pont*), suggested by O'Brian and Hance (1940), is probably the best. With immersion objectives, sandalwood or Shillaber oil may be employed as the immersion medium. No special objectives or oculars are required; however, Jenkins (1937) has pointed out that some of the older objectives contain balsam that gives rise to its own fluorescence, and in these cases a darkfield stop must be used in the condenser to prevent the entrance of direct ultraviolet rays into the objective.

A filter (*EF*) that excludes ultraviolet, and passes visible rays, is placed on the ocular. Either the Corning filters No. 3389 or 3060, the Leitz No. 8547A, the Bausch and Lomb or the Zeiss Euphos filter, or a circle of Wratten 2A gelatin filter cut to fit inside the eyepiece may be used. Metcalf and Patton (1944) recommend a 5% solution of sodium nitrite contained in a plane-sided glass cell 5–10 mm. in optical depth which may be conveniently placed on the diaphragm of the ocular or, better still, on the diaphragm of the microscope tube.

2. Preparation of Tissues

The preparation of sections from frozen dried material has the advantage that soluble or diffusible constituents will have no chance of being lost or displaced from their original sites. However, while this is the method of choice, paraffin sections of formalin-fixed tissue have been employed with success in certain instances, although neither celloidin nor gelatin sections can be used since these media give rise to fluorescence.

When paraffin sections are employed, fixation is usually carried out in 5–10% formalin for not longer than 24 hr. The sections are cut 7–8 μ thick; egg albumin has been used to make them adhere to the slides. The paraffin is removed by immersion in xylol for 30 min. and the sections are dried at room temperature. In this form they have been kept for months, and may be examined without further treatment. All reagents and materials should be the purest obtainable to avoid adventitious fluorescence of contaminants.

3. Photomicrography

Photomicrographs of fluorescing preparations can be made if certain precautions are taken. Popper and Elsasser (1941) found that, in the photomicrography of vitamin A fluorescence, it is preferable to use film of maximal daylight sensitivity. The Fluorapid film of *Agfa-Ansco Corp.* is particularly well suited for the purpose (Fig. 4C,D). Kodachrome film can be used when the tissue is rich in vitamin A, but the lower sensitivity of this film limits its value. In general black-white film is preferable to the color variety.

With substances of fading fluorescence, such as vitamin A, the time employed for focusing must be kept to a minimum even at the expense of the sharpness of the picture. The exposure itself must be short (a maximum of 30 sec., regardless of magnification, in the case of vitamin A) in order to obtain greater contrast between the fluorescence and the background. Only one exposure can be made as shown in Figure 4A,B.

When the fluorescence does not fade, Kodachrome film can give excellent results. The exposure time has to be determined by trial in each case, usually falling in the range of 1–15 min. with an average of about 2 min., according to Metcalf and Patton (1944). The loss in intensity with greater magnifications makes it impracti-

cal to employ magnifications exceeding 500 times. Metcalf and Patton (1944) also report that with black-white film having a Weston rating of 50, exposures of from 2 sec. to 5 min., with an average time of about 10 sec., are required with a 35 mm. camera. In all fluorescence photomicrography the dark-field stop on the

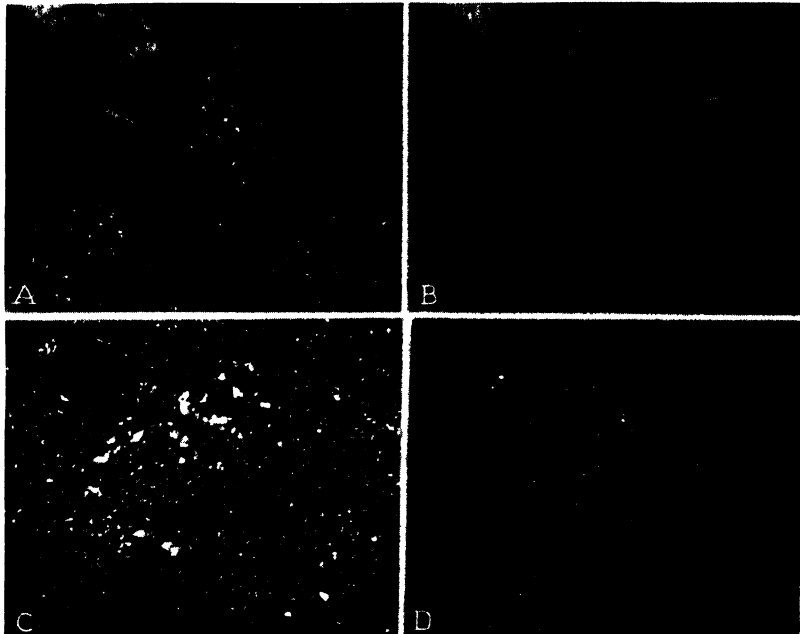



Fig. 4. A, Human liver showing collagenous fibers in the periportal field and vitamin A fluorescence in the liver cells. B, Second exposure of the same field; the vitamin A fluorescence has faded. C, Rat liver photographed with sensitized film (Fluorapid); a large amount of vitamin A fluorescence in the Kupffer and the liver cells is evident. D, Picture of a liver taken with normal ultraspeed film. *From Popper and Elsasser (1941)*

condenser and the filter placed on, or in, the eyepiece or microscope tube to screen out ultraviolet rays must be used. Otherwise fogging may occur from the stray radiation. Metcalf and Patton (1944) especially recommend the sodium nitrite filter for color photography. In order to switch from ultraviolet to visible illumination a piece of opal or ground glass is substituted for the filter placed in front of the light source.

4. Characterization of Substances

Direct Observation of Fluorescence

Vitamin A. Particularly intensive work has been done on vitamin A, starting with the work of von Querner (1935), Hirt and Wimmer (1940), and others, and continuing in greatly expanded scope and development with the research of Popper (1944) and associates. The fading green fluorescence of vitamin A in tissue sections has been found to run parallel with the results of chemical determination (Popper and Elsasser, 1941). The fading green fluorescence is characteristic of vitamin A_1, found in salt water fish, and a slowly fading pale yellow-brown fluorescence characterizes vitamin A_2, found in fresh-water fish. An admirable review of the studies made on vitamin A distribution in the tissues of animals and man, both in normal and pathological states, has been presented by Popper (1944). He pointed out that carotene may easily be differentiated from the A vitamins by its very slowly fading green fluorescence which is apparent only in higher concentrations, and that the biologically inactive anhydro ("cyclized") vitamin A may be recognized by its dark brown fluorescence which gradually becomes a dull green and finally fades out entirely. Volk and Popper (1944a) reported the existence of a factor in biological fluids, particularly plasma, and in organ emulsions that delays the disappearance of vitamin A fluorescence in tissue sections.

Riboflavin. Ellinger and Koschara (1933), von Euler *et al.* (1935), Hirt and Wimmer (1939a), and Metcalf and Patton (1942) utilized the yellowish-green fluorescence of riboflavin for its identification in tissues. According to Ellinger (1938), and confirmed by Metcalf and Patton (1942), another form of riboflavin exists (probably bound to another compound) which gives a yellow-orange fluorescence. Metcalf (1943) subsequently concluded that in the American roach, *Periplaneta americana* L., the bound riboflavin is converted to the free form *in vivo* by the injection of pantothenic acid or thiamine. With the latter compound the conversion proceeds more slowly.

Other Vitamins.* Attempts have been made to characterize, and to determine the distribution of, other vitamins by their fluorescent properties. Hirt and Wimmer (1939b) investigated nicotinic acid and its amide which they claimed gave a stable yellow fluorescence.

* See Bibliography Appendix, Ref. 29.

They reported that in the dry state the amide has a weaker fluorescence than the acid, but that in a 1% aqueous solution the reverse is found. Ellinger (1940) was not able to observe fluorescence with purified solutions of these compounds, and he also disagrees with Hirt and Wimmer (1939b) that ascorbic acid can be detected microscopically by fluorescence. The histochemical opportunities of studying vitamin K by means of its well-known fluorescence are obvious. One may expect that studies of this nature will be made in the future.

Lipids. During the course of his work on vitamin A, Popper (1941) also studied the histological detection of lipids by means of the fluorchromes: methylene blue, thioflavin S, rose bengal, magdala red, and phosphine 3R. The last stain appeared to be the best. Further examination revealed that fatty acids, soaps, and cholesterol are not made apparent by phosphine 3R which, however, does visualize neutral fat as a silver-white fluorescence on a brown background (Volk and Popper, 1944b; Popper, 1944). The advantage claimed for this method is that, because of the water solubility of the dye, more and finer droplets of lipid can be detected than would be possible by the usual stains. Popper (1944) recommends the use of a 0.1% aqueous solution of phosphine 3R (*Pfaltz and Bauer*) for 3 min. on frozen sections of tissue.

Pigments. The fluorescence technique has been applied to studies of certain biological pigments. Thus the red fluorescence of porphyrins has been employed in histological studies of these compounds (Lison, 1936, page 256; Ellinger, 1940; Dobriner and Rhoads, 1940; Grafflin, 1942). Chlorophyll has been localized microscopically in plant tissues by Tswett (1911) and Wilschke (1914) by means of its red fluorescence. The fluorescent properties of bile pigments in the presence of zinc acetate, and of uropterin, might be adapted to microscopic studies of these pigments.

Carcinogenic Hydrocarbons. Investigations of carcinogenic hydrocarbons in tissues have made use of the fluorescence of certain of these compounds. Graffi (1939, 1940) investigated the distributions in normal and tumor cells of pyrene, benzpyrene, anthracene, dibenzanthracene, and methylcholanthrene, while Gunther (1941) and Doniach *et. al.* (1943) confined their studies to benzpyrene, and Simpson and Cramer (1943–1945) explored the histological localization of 20-methylcholanthrene in skin.

TABLE I

Fluorescence Color of Various Tissues after Heating to Different Temperatures (Helander, 1945b)[a]

Structures and tissues	Before heating	150°	175°		200°		225°		250°		300°
		5 min.	3 min.	5 min.	3 min.	5 min.	3 min.	5 min.	3 min.	5 min.	3 min.
Elastin	bl.	bl.	bl.	p.bl.	p.bl.	p.bl.	bl.-w.	bl.-w.	g.-w.	y.-w.	0
Collagen	d.bl.	d.bl.	d.bl.	d.bl.	bl.	bl.	p.bl.	bl.-g.	p.-g.	p.-g.	0
Fat tissue	bl.	g.-w.	y.-g.-w.	y.-g.-w.	y.-g.-w.	y.-w.	y.-w.	g.-y.	g.-y.	g.-br.	0
Muscles											
Skeletal	bl.-w.	bl.-w.	bl.-g.; w.-g.	bl.-g.; w.-g.	w.-g.	y.-g.	y.-g.	y.-g.	d.y.-g.	g.-br.	0
In skin	y.-g.	y.	y.	y.	y.	y.-br.	y.-br.	y.-br.	y.-br.	br.	0
Cardiac	bl.-g.	bl.-g.	bl.-g.	bl.-g.	bl.-g.	p.bl.-g.	g.-y.	y.-br.	g.-br.	d.g.-br.	0
Smooth	bl.-g.	y.-g.	g.-y.	g.-y.	g.-y.	g.-y.	y.	y.-br.	g.-br.	d.g.-br.	0
Nerves	Intense bl.	bl.-w.	bl.-w.	g.-w.	g.-y.	g.-y.	y.-g.	y.-g.	d.y.-g.	y.-br.	0
Ganglion cells											
Cytoplasm	p.bl.-g.	p.g.	p.g.	p.g.	g.-w.	g.-w.	w.-y.	g.-y.	g.-y.	y.-br.	0
Nuclei	d.bl.	d.bl.	d.bl.	bl.	g.-w.	g.-w.	y.-w.	y.-w.	y.-w.	y.-br.	0
Resp. epithel. cells											
Cytoplasm	g.-w.	g.-w.	y.-w.	y.-w.	y.-w.	y.-g.	d.y.-g.	d.y.-g.	y.-br.	d.br.	0
Nuclei	d.g.	d.g.	p.g.	p.g.	p.g.	p.g.	y.-g.	y.-g.	g.-br.	d.br.	0
Kidney tubule cells											
Cytoplasm	bl. and g.	bl. and g.	bl. and g.	p.g.-bl.	p.g.-bl.	y.-g.	y.-g.	g.-y.	g.-y.	y.-br.	0
Nuclei	d.bl.	d.bl.	d.bl.	g.-bl.	g.-bl.	g.-bl.	y.-w.	g.-y.	g.-y.	y.-br.	0
Spleen cells											
Cytoplasm	bl.	p.bl.	p.bl.	p.bl.	p.bl.	g.-w.	y.-g.	g.-y.	g.-y.	br.-g.	0
Granules	y.	y.-br.	y.-br.	0	0	0	0	0	0	0	0
Liver cells											
Cytoplasm	bl.	bl.-g.	g.	g.	g.-y.	y.-g.	y.-g.	y.-g.	y.-g.	d.g.-br.	0
Nuclei	d.bl.	p.bl.	p.bl.	p.bl.	p.bl.	g.-bl.	g.-w.	g.-y.	g.-y.	br.-g.	0
Intestine cells											
Cytoplasm	p.bl.	p.g.	p.g.	p.g.	g.-y.	g.	y.-g.	g.-y.	g.-y.	g.-br.	0
Nuclei	d.bl.	d.bl.	p.bl.	p.bl.	p.bl.	p.bl.	g.	g.-y.	g.-y.	br.-g.	0
Red corpuscles	0	0	d.g.-br.	g.-br.	g.-br.	g.-br.	br.-y.	br.-y.	y.-g.	d.br.	0

[a] Abbreviations: bl. = blue, br. = brown, g. = grey, w. = white, y. = yellow, d. = dark, p. = pale, 0 = no fluorescence.

TABLE II

Fluorescence Color of Various Drugs and Change in Color of Tissue Sections after Heating (Helander, 1945b)[a]

Drugs	Melting points, °C.	Fluorescence in pure, pulverized form[b]		Fluorescence in tissues										Concn. of injected solns.
				Before heating		After 150° for 5 min.		175°, 5 min.		200°, 5 min.		225°, 5 min.		
		Color	Intensity	Color	Contr.	Color	Contr.	Color	Contr.	Color	Contr.	Color	Contr.	
acid	190–2	bl.	+	—	—	br.	+++	br.	+++	y.-br.	+++	y.-br.	++	1:100
oline (*Roche*)	172–3	p.bl.	++	—	—	y.	+	y.	+++	y.	++	y.-br.	++	1:4000
sulfate	181–3	bl.	++	—	—	br.-y.	+++	br.-y.	+++	r.-v.	++++	p.r.	+	1:100
bitartrate	146–8	gr.	+++	—	—	—	—	g.-br.	++	y.-br.	+++	br.-y.	++	1:100
	125	y.	+	—	—	br.-g.	+	br.-g.	+++	br.-g.	++	y.-br.	+	1:100
	270	—	—	—	—	y.	+	y.	+++	y.	+++	y.	++	1:100
ine	158	gr.	++++	gr.	++	gr.-y.	+++	g.-y.	++	g.-y.	+	p.br.	+	1:100
pine hydrobromide	212	—	—	—	—	br.	+	br.-y.	++	y.	+++	y.	+++	1:100
n	149–50	bl.	+++	—	—	y.	++	y.	+++	y.	+++	y.-br.	++	1:100
ne hydrochloride	231	v.	+	p.-gr.	+++	p.gr.	+++	br.-y.	+++	br.-y.	+++	br.-y.	++	1:100
ı	—	gr.-y.	+++	—	—	y.	+	y.-br.	+++	y.-br.	+	br.	+	1:1000
(*Ciba*)	97	bl.	++	—	—	y.-g.	+	y.-g.	+++	y.-g.	++	y.	+	1:100
Ciba)	171	—	—	—	—	—	—	y.	+++	y.	++	y.	+	1:1000
l soln. (*Bayer*)	—	r.	+++	r.	++++	r.	+++	r.	++	r.	++	r.	++	1:1000
in	275–80	y.	+++	gr.	+++	gr.-br.	++	br.	+++	br.	+++	br.	+	1:1000
rine (*Pharmacia*)	—	r.-br.	+	g.-br.	+++	g.-br.	+++	br.-g.	+++	br.-g.	++	g.-y.	+	1:1000
iazole (*Pharmacia*)	—	r.-br.	+	d.br.	+++	d.br.	+++	p.br.	+++	p.br.	+++	r.br.	+++	1:1000
salicylate	—	bl.	++++	bl.	+	bl.	+++	bl.	+++	bl.	+++	br.-g.	+	1:100
mide (*Astra*)	163	bl.-g.	+	—	—	y.	+++	y.	+++	y.-br.	++	br.	++	1:1000
idine (*Pharmacia*)	190–3	bl.	++++	—	—	y.	+++	y.	+++	y.-br.	++	br.	++	1:1000
zole (*Astra*)	202	bl.	+	—	—	—	—	y.	+++	p.br.	+++	d.br.	+++	1:1000

eviations: bl. = blue, p. = pale, br. = brown, contr. = contrast, g. = grey, gr. = green, r. = red, v. = violet, y. = yellow.
distinctness of the contrast is denoted by +. The greater the number of + signs, the more distinct the contrast color.

Chemotherapeutics. Helander (1945a) has reported the detection of chemotherapeutics in paraffin sections prepared from frozen dried tissue. The blue fluorescence of tissue itself, when illuminated by ultraviolet, may obscure the fluorescence of compounds present if they too have a blue fluorescence. This difficulty can be circumvented in some cases by heating the sections for a short period in order to change the color of the fluorescent light emitted by the drug. In a work of a high order of excellence Helander (1945b) presented a table (Table I) of the fluorescences of various tissues, and after intramuscular injection of drugs into mice, he found that sections of muscle tissue showed the fluorescences indicated in Table II.

Fischl and Singer (1935) made use of the fluorescences of atebrin and trypaflavin to show that these compounds are taken up *in vivo* by trypanosomes. A table of the fluorescences of compounds, and of certain parasites after treatment with them, has been given in a paper by Bock and Oesterlin (1939).

Metals. The detection of uranium salts in incinerated sections of tissue has been made possible by the fluorescence of these salts, (page 145).

The colors of the insoluble 8-hydroxyquinoline derivatives of iron, calcium, magnesium, aluminum, manganese, zinc, and copper have been used for identifications of these metals in tissue sections (see page 21). However, no one seems to have exploited the possibility of utilizing the fluorescences of these derivatives. DeMent (1942) lists the fluorescent colors of 27 metallic hydroxyquinolinates, and it would appear that, in some cases at least, a more clear-cut differentiation between the metals would be possible on this basis.

Spectroscopic Analysis of Fluorescence

The identification of substances in tissues by means of the spectroscopic analysis of the fluorescent light they emit has been utilized to some degree. Dhéré (1939) presented a thorough and able review of this technique and gave data relative to various carbohydrates, lipids, proteins and amino acids, alkaloids, chlorophylls, bile pigments, porphyrins, carotenoids, flavins, thiochrome, and a few other special compounds. Either direct vision or grating microspectroscopes may be employed, and the spectral lines of mercury can be

used conveniently for calibration after the filters are removed, since a mercury arc is the source of the radiation.

More recently Sjöstrand (1946a,b) has employed this technique for the localization of riboflavin and thiamine in cells. Sjöstrand used the freezing-drying treatment on this tissue (frog eyes, etc.) followed by paraffin infiltration. The 3–5 μ sections were subjected to ultraviolet illumination on the stage of a microscope and the fluorescent light which resulted was passed from the ocular into a spectroscope for analysis.

B. EMISSION HISTOSPECTROSCOPY

The technique for the identification of certain elements in selected histologically defined portions of tissue by means of emission spectra was developed independently and at about the same time by Gerlach and Gerlach, and by Policard. Modifications were made later chiefly by Scott and Williams. The emission spectra are obtained from the radiation produced by consuming, in high-frequency spark, a chosen cellular region of a tissue section or a selected bit of biological material. The amount of tissue destroyed in sections is a disc of about 1 mm. diameter and 0.1 mm. depth; this would have a weight of approximately 0.08 mg. and hence elements giving good spectral lines can be detected in this quantity of fresh or fixed tissue. Elements that have been identified by this technique include potassium, sodium, calcium, magnesium, manganese, aluminum, iron, zinc, lead, copper, mercury, silver, gold, carbon, silicon, phosphorus, and boron. Attempts at quantitative work have been made, but the technique is primarily qualitative at present.

There is little essential difference between the apparatus developed by Gerlach and Gerlach (1933) and Policard (1931–1932, 1933a). The former employed a high-frequency generator that was superior to the one used by Policard, and the latter used a metal slide to hold the tissue section instead of the glass slide used by the Gerlachs.

1. Policard Technique

A frozen section, 50–200 μ thick, cut preferably from fresh tissue or else from alcohol or formalin-fixed material, is placed on a slide of pure platinum or gold which is fastened to the microscope stage

(Fig. 5). A platinum or gold needle embedded in a cylinder of insulating material, which is fixed in one of the objective holes of the microscope's revolving nosepiece, serves as a movable electrode. The circuit is completed through the frame of the microscope which is grounded. The cellular region to be examined is chosen with an objective in position, and then the objective is swung out and the electrode needle brought into place. The needle should be in line with the optical axis of the microscope so that it is centered over the selected spot. The distance between the point of the needle and the section is fixed between 1–2 mm. When the circuit is closed, the radiation from the spark produced is condensed by the quartz lens and directed through a 0.1–0.2 mm. slit into the quartz spectrograph. For a strong spark, a period of not more than 1 sec. seems to be sufficient for the exposure of the photographic plate on the spectrograph, but for weak sparks 20–30 sec. may be required according to Policard. The best spectral lines were observed in the ultraviolet range, 100–300 mμ.

Fig. 5. Arrangement of Policard's apparatus for emission histospectroscopy: 1, Microscope stage under revolving nosepiece bearing the upper electrode; 2, high-frequency apparatus—dermatological type; 3, Hilger quartz spectrograph; 4, cylindrical quartz condensing lens; 5, optical bench; 6, ground lead; 7, current source; 8, upper electrode lead. *From Policard (1931–1932)*

2. Scott and Williams Technique

Scott and Williams (1935) modified the apparatus chiefly by screening the electrodes, placed 1.4 cm. apart, in order to practically eliminate electrode lines from the spectra and to obviate the need of cleaning the electrodes often. Since these workers did not employ needle electrodes mounted on a microscope, they could not make as fine a histological selection of the sample as the European investigators. Scott and Williams (1934) minimized this point and claimed that removal by fine dissection of the bit of material to be studied is sufficient. In most instances this would be true, but there are

conditions conceivable in which the needle electrode assembly might have advantages. In any case the Scott and Williams apparatus has a number of significant improvements, as will be apparent from the following description of it.

The spark generator construction is shown diagrammatically in Figure 6. Oscillations are produced in the primary circuit by a spark passing between two thick brass discs rotated by a small motor to prevent erosion and local heating. The space between the discs is supplied with photoelectrons by focusing ultraviolet radiation on it from a quartz mercury lamp. The analyzer spark in the secondary circuit has an improved continuity and steadiness as a

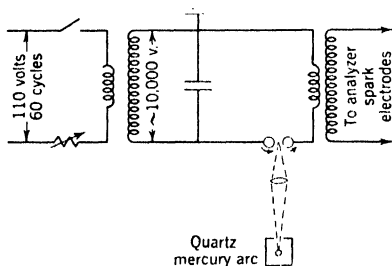



Fig. 6. Wiring diagram of spark generator assembly. The ionizing beam of ultraviolet radiation is shown focused between the rotating discs of the spark gap. *From Scott and Williams (1935)*

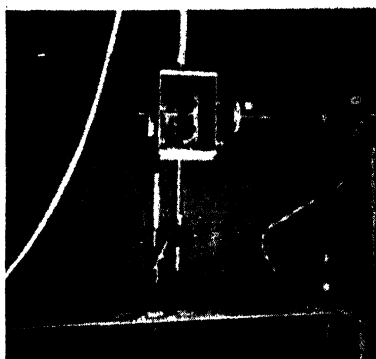



Fig. 7. View of box enclosing analyzer spark gap and electrode screen. Portion of spectrograph seen at right. *From Scott and Williams (1935)*

result. A large open type oscillation transformer is employed having two heavy primary, and twenty smaller secondary, turns wound on a wooden frame as close to the edge of a 36 in. square as possible. A clearance of 0.5 in. allowed between the secondary turns. The condenser is composed of a stack of glass plates ($8 \times 8 \times 1/8$ in.) separating eight sheets of aluminum foil (6×6 in.) connected alternately. The current passing over the analyzer spark gap is about 0.45 ampere.

The electrodes of the analyzer spark gap are steel balls (0.25 in. diameter) placed 1.4 cm. apart which are supported through the bakelite back of a metal box housing fitted with quartz and glass

windows, removable for cleaning. An aspirator tube is fitted into the top of the box to remove vapors and the box itself is placed on alignment pegs to maintain fixed optical relations. The disposition of the spark gap relative to the spectrograph is shown in Figure 8. As mentioned previously, the horizontal slit in the side of the box facing the spectrograph serves to screen out electrode lines from the tissue spectra and to obviate the need of cleaning the electrodes oftener than once a month. The end of a 2 in. length of Pyrex tubing (2–3 mm. inside diameter) or rod (2–3 mm. diameter) is centered in the spark gap. It has been shown that the glass does not affect the spectra. Bits of cellular material (3–6 μl.) are placed on the end of the glass so that they will be in the center of the spark. A

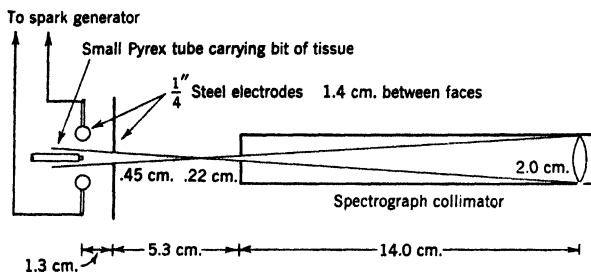



Fig. 8. Diagram of disposition of the spark gap relative to the spectrograph.
From Williams and Scott (1935)

film of purified Eastman gelatin may be helpful in effecting the adherence of dry material. A strip of tissue, 5 mm. or more long, may be placed in the glass tube and fed into the spark with a push rod; and in a similar manner liquid taken up on a small strip of ashless filter pulp can be subjected to test. In the latter case, control spectra for the pulp alone must be obtained.

A Gaertner L250W quartz prism spectrograph taking 3¼ × 4¼ in. photographic plates was used. The spectrograph slit was fixed at 0.05 mm., and Eastman "50" plates were employed for high sensitivity and Eastman Process plates for high contrast. Thirty exposures may be made on each plate. Two microscopes with a comparison ocular were utilized for comparing the positions and intensities of lines on different plates.

Faint iron lines from the electrodes are apparent in the spectra

so that, if iron is to be investigated, another electrode metal should be used. For an adequate exposure (15–30 sec.), 2–4 μl. of tissue are usually sufficient. When tissue is subjected to fixation, a control experiment is necessary to test the fixing fluid spectrographically; the filter pulp method may be employed for this purpose.

The Williams and Scott (1935) photoelectric apparatus for dark-field photometry and densitometry has been used to determine the intensity of the spectral lines in order to obtain a more quantitative estimation of the elements. A description of this apparatus is given in the section dealing with microincineration (page 146). The blackness of the photographed spectral lines is measured by placing the photographic plate on the mechanical stage, adjusting the reflecting prism so that the light emerging from the ocular is reflected directly downward on the slit of the photocell box, and observing the galvanometer deflection after the proper focusing has been made. The deflection for an unexposed portion of the photographic plate is then taken.

C. VISIBLE AND ULTRAVIOLET ABSORPTION HISTOSPECTROSCOPY*

The application of the quartz microscope to measurements of the absorption spectra of cellular components *in situ,* particularly as developed and applied by Caspersson and co-workers at Karolinska Institutet, Stockholm, offers a new and promising approach to the solution of many histo- and cytochemical problems. The ingenious apparatus of Caspersson (1940), subsequently modified by Gersh and Baker (1943), has already yielded valuable information concerning the nucleic acids of chromosomes (Mirsky, 1943), the nature of thyroid colloid (Gersh and Baker, 1943), and chemical characteristics of the Nissl bodies in nerve material (Gersh and Bodian, 1943-a,b). (It should be pointed out that the absorption method cannot differentiate between ribonucleic acids and desoxyribonucleic acids since their absorption spectra are almost identical, having maxima at about 260 mμ. However, the differentiation can be made qualitatively by staining reactions, (pages 65, 66).

The great advantage of studying cell structures *in situ* by this technique is made particularly impressive by the fact that the spectral measurements can be carried out on quantities of material

* See Bibliography Appendix, Ref. 31.

down to 10^{-8} μg. Investigations may be made on selected structures in microtome sections or on mechanically separated cellular components. When sections are to be employed, they are best prepared from tissue embedded in paraffin or celloidin after freezing-drying treatment. The technical requirements in absorption spectra measurements by means of the ultraviolet microscope have been examined critically by Cole and Brackett (1940). Lavin (1943) simplified the focusing of the ultraviolet microscope by using a willemite screen which produces a visible image with ultraviolet illumination.

The absorption technique applied *in situ* has certain drawbacks that should be considered, elegant though the technique is. Thus, Danielli (1946a) has sounded a warning that hazards exist in ascribing to particular substances the absorptions found in different parts of a cell. Effects of molecular interactions and interferences by other substances are possibilities that are not to be ignored. Hence the method will be of greatest value when the results are interpreted with appropriate regard to these limitations.

A most ingenious microscope arrangement for the colorimetry of 0.5–1.0 μl. drops of liquid was developed by Norberg (1942), also at Karolinska Institutet, and applied by him to the measurement of phosphorus in quantities down to 0.5 mμg. This technique requires the removal of the specimen from the rest of the tissue and its chemical treatment to yield a solution which can be subjected to absorption analysis.

Stowell (1942) designed an apparatus for the measurement of the amounts of stain or pigment in tissue sections. For the measurement of stained constituents the quantitative significance of the method depends on the degree of correlation between the amounts of the stain and the substance for which the stain is specific, a correlation often poorly defined. Stowell applied his technique to the estimation of desoxyribonucleic acid by means of the Feulgen stain.

1. Caspersson *in Situ* Technique

A diagrammatic representation of the apparatus is given in Figure 9. The source of radiation may be either the Philips water-cooled super-high-pressure mercury lamp (*A*), a tungsten lamp (*B*) supplied with current from storage batteries, or Köhler's rotating electrode spark gap (*P*). The mercury lamp may be used for radiation in the visible portion of the spectrum and in the ultraviolet range down

to 230 mμ. However, since variations both in voltage and water pressure affect the mercury lamp, the tungsten is employed for the longerwave ultraviolet range. The spark source is used for wavelengths shorter than 235 mμ.

The radiation, after passing through a monochromator (*C*) is concentrated on the object (*I*) on a quartz slide by the condenser (*H*). (The second monochromator slit is at *D*, lens at *E*, and a 90° quartz prism at *F*.) For ultraviolet work a fused quartz condenser is used, and in the visible range a good achromatic type is employed. Zeiss objectives designed for the longer ultraviolet (down to about 340 mμ) or the fused quartz objectives of Köhler and von Rohr for the shorter wavelengths are used (*K*). For routine work down to

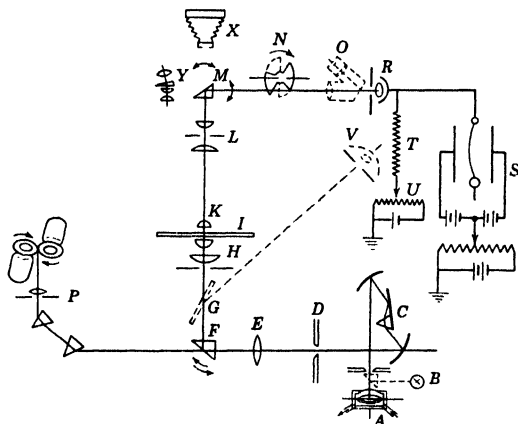



Fig. 9. Arrangement of Caspersson's apparatus for photoelectric absorption histospectroscopy. *From Caspersson (1940)*

240 mμ, Caspersson (1940) found it convenient to have a glycerin immersion lens corrected for 257 mμ with an aperture of 1.25, one corrected for 275 mμ, and a long-wave ultraviolet recorrected apochromat. Oculars with iris diaphragms (*L*) were employed. The lenses in them were of quartz for the ultraviolet work.

A 90° quartz prism (*M*), adjustable by means of micrometer screws, is used to deflect the radiation through the opening of an electrically driven rotating sector (*N*) on to a photoelectric cell (*R*). The prism can be replaced either by a Köhler focuser (*Y*) or

a photomicrographic camera (X). Every object measured must be photographed in order to establish its exact position and dimensions. The photocell is connected to a string electrometer and both of these instruments are well shielded and also protected from moisture by means of phosphorus pentoxide. Various photocells are used for different wavelengths; gas cells are usually employed. For the shortest ultraviolet, cadmium; for medium ultraviolet (260–350 mμ), sodium; for long ultraviolet and visible (350–550 mμ), potassium; and for wavelengths over 550 mμ, potassium-cesium cells are used. The telescope (O) is placed in front of the photocell to control the optical centering of the system; this centering must be very exact.

It is necessary to compensate for variations in the source of intensity of the radiation, and for this purpose a quartz plate (G) is interposed in the optical path in order to reflect a small percentage of the radiation on a photocell (V). Readings of the changes in the photocell current can be used to correct the readings of the electrometer (S). (T and U are leak resistance and four-step potentiometer, respectively.)

Measurements are made by taking the deflection of the electrometer with the object in position and in focus, and then moving the object away so that a clear space on the slide lies in the optical axis. The opening in the rotating sector is reduced until the amount of radiation striking the photocell is the same as before, *i.e.*, the same electrometer deflection is produced. The absorption in the object will then be equal to the decrease effected by the sector, and extinction coefficients may be calculated.

Gersh and Baker Modification. A somewhat simplified set-up, with American-made instruments, is employed by these investigators, as may be seen in the diagram of their apparatus (Fig. 10).

The source of radiation is a Daniels and Heidt (1932) type of medium pressure mercury arc in a quartz capillary tube which is mounted about 1 cm. from a quartz window in a large copper box. The lamp is water cooled, and since the rate of cooling affects the radiation output, the water line is equipped with a pressure regulator. The lamp consumes 500–700 watts from a 220 volt D.C. line; a ballast resistance is placed in series with the lamp.

The entrance opening of the monochromator is a circular hole of about 0.8 mm. diameter in a thin sheet of copper fixed 0.5–1.0 mm. in front of the arc, in the water bath. The two equilateral quartz

prisms of the monochromator are 5 cm. long and 4 cm. high; the table on which they are mounted can be rotated by means of a slow-motion screw. The collimating lens has a focal length of about 8 cm. and the telescope lens about 80 cm.; both are held in adjustable brass mountings and these with the prism table are fastened to an iron plate on leveling screws. The whole apparatus is enclosed in a wooden box with the required apertures. A spectrum from the first

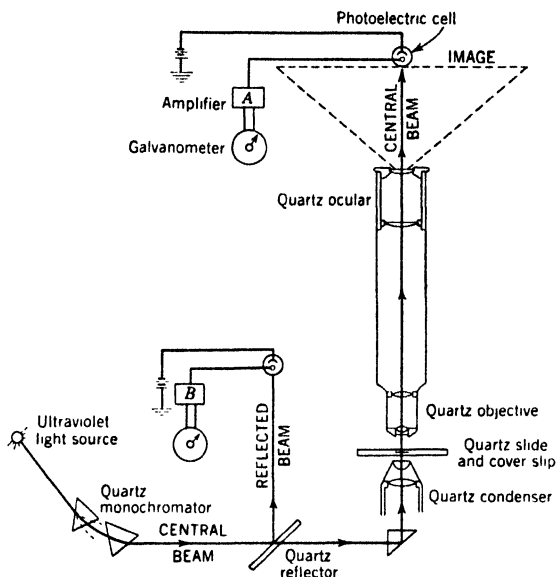



Fig. 10. General arrangement of light source, monochromator, microscope, and measuring device for determining the absorption of ultraviolet light by minute volumes of tissue. ***From Gersh and Baker (1943)***

prism, without entering the second, passes out of one of these apertures to fall on a calibrated wall scale 2 meters distant. By this means the prisms can yield any chosen wavelength when manually adjusted.

A Zeiss quartz microscope of Köhler design is mounted on a leveling table so that it can receive the radiation reflected by a quartz right-angle prism. In order to fill the objective field, the substage condenser is focused on the field of the telescope lens rather

than on the image of the entrance opening of the monochromator. The diameter of the condenser diaphragm is set at 6 mm. to insure sufficient spectral purity.

Both photoelectric and photographic recording may be employed. The quartz photoelectric cell is a No. FJ-405 of *General Electric Co.*, and it is mounted 56.5 cm. above the ocular in a large brass cylinder. This cylinder has a quartz window close to which is a frame that holds a series of circular slits. The cylinder is horizontally adjustable so that a slit can be brought into the optical axis of the microscope. The brass cylinder also contains a type D96475 electrometer tube of *Western Electric Co.*, and a 10^{10} ohm SS White grid resistor. The output from the photocell is connected to the grid of the tube which is included in a Penick amplifier circuit maintained on three storage batteries of large capacity. The amplified current is measured by a Leeds and Northrup type R galvanometer with a scale 1.5 m. from the galvanometer mirror.

In order to bring the photocell into adjustment in the optical axis of the microscope, the shadow of an ocular cross hair is projected by means of "white" light on the photocell aperture, which is then adjusted until its center and the center of the image coincide. The cross hair in a fluorescent finder placed above the ocular is adjusted similarly with "white" light and ultraviolet radiation.

For the measurement of absorption curves of larger uniform objects, the object is centered in the cross hair of the finder, the condenser is focused on the plane of the telescope lens, the objective is adjusted to give a sharp image, and the current generated in the photocell is measured. Then the object is moved away so that only the clear slide is in the optical path and another measurement is made. From these data the percentage transmission and the extinction coefficient can be calculated. The measurements are then repeated at each wavelength chosen. For studies of thyroid colloid, Gersh and Baker (1943) used a 6 mm. objective, 10$\times$ ocular, and a photocell aperture of 11.9 mm. With these optics the light transmission was measured through a tissue area of 143 μ^2 and a volume of 2861 μ^3. For measurements on cytoplasm and nucleoli the respective systems were 6 and 1.7 mm. objectives, 14 and 10$\times$ oculars, and 8.73 mm. photocell aperture in both cases.

When the measurements are to be made on smaller and less homogeneous objects such as Nissl bodies, a different and more

accurate technique is used. A quartz plate 1 mm. thick and 2 cm. square is placed in the path of the monochromatic beam at an angle of 45° and at a distance from the telescope lens of 46 cm. (Fig. 10). By this means 6–7% of the incident radiation is reflected to another photocell mounted at a distance from the quartz plate equal to that of the aperture of the microscope condenser. The photocell output is amplified by a Huntoon (1935) direct-current amplifier and passed through a Leeds and Northrup type P wall galvanometer. Simultaneous readings are taken on the same scale, from both this galvanometer and the one used for recording transmission, at each wavelength without moving the object, but with careful focusing of condenser and objective at each wavelength. After these readings are obtained the object is moved away and the readings for the clear quartz slide are taken. The data are used to calculate the transmission and extinction coefficients as usual.

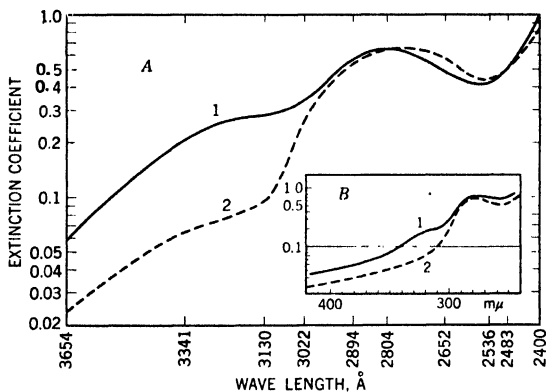



Fig. 11. Absorption curve of colloid in a single follicle of a thyroid gland in alkaline (*A*1) and acid (*A*2) medium, as compared with the ultraviolet absorption curves of extracted sheep thyroglobulin made by Ginsel in alkaline (*B*1) and acid medium (*B*2). *From Gersh and Baker (1943)*

The reliability of the technique is shown by Figure 11, taken from Gersh and Baker (1943). The curves in the inset (B) were obtained by Ginsel (1939) for extracted sheep thyroglobulin in both acid and alkaline media, while those in (A) were established by Gersh and Baker by their histospectrographic technique on the colloid in a single thyroid follicle.

2. Norberg Technique

Apparatus

A diagram of the optical system is given in Figure 12. For work in the visible range, the light source employed is a 100 watt tungsten band lamp (*A*) supplied with current from a large capacity (150–200 amp. hr.) storage battery. Monochromatic light is obtained from a Winkel-Zeiss monochromator (*B*). (*C* is second monochromator slit.) A filter (*F*) may be used between the condenser (*E*) of the microscope and the sample slide (*G*). The microscope objective is indicated by *H* and the ocular by *O*. During measurement the ocular is removed and the light is reflected by the prism (*I*), which is mov-

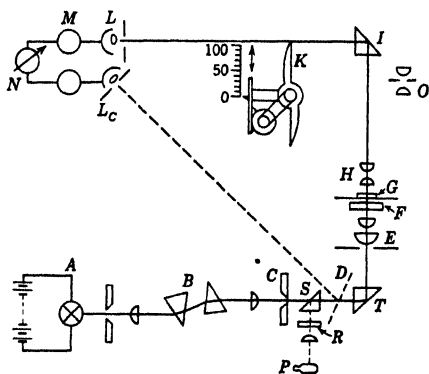



Fig. 12. Microphotometer. *From Norberg (1942)*

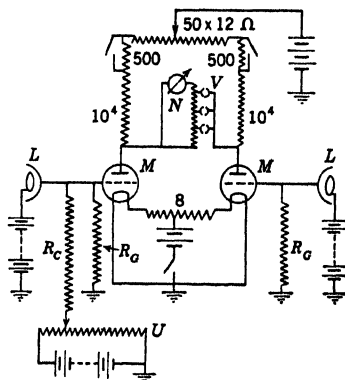



Fig. 13. Photocell amplifying circuit. *From Norberg (1942)*

able about both a horizontal and vertical axis, to the photocell (*L*). Potassium cells are employed for wavelengths 450–550 mμ, and potassium-cesium cells for longer wavelength. The current generated in the photocells is amplified by circuits in *M* and then conducted to the galvanometer (*N*). Details of the amplifying circuit will be considered subsequently.

For measurements in the ultraviolet region a high-pressure mercury lamp (P) with movable prism (*S*) and 90° prism *T*, of the Philips Philora H P 300 type, is used with a spectral filter (*R*). Variations in the intensity of the radiation from the mercury lamp

are compensated by casting a portion of the radiation on photocell L_c by means of the semireflecting glass (D). The current generated in this photocell is amplified and made to oppose that from photocell L.

The galvanometer is employed as a null-point instrument and the amount of radiation striking the photocell L is controlled by the rotating sector (K). The rotating sector with its motor is mounted on a slide, adjustable by a rack and pinion arrangement. The radiation passing the sector can be controlled over a greater range (0–50%) by the use of two discs, each with a 90° segment removed, which are made to rotate in opposite directions. Kortüm (1934) has described a simple sector which operates on a similar principle. Norberg finally employed a sector patterned after the Askania-Werke (Berlin) model, which enables adjustment and reading while the sector is in action, and the accuracy obtained in the absorption is 0.025%.

The amplifying circuit for the photocell current is shown in Figure 13. It is a modification of that described by Custers (1933) and it utilizes two Philips 4060 electrometer tubes. The apparatus can be used with either the single photocell (L, Fig. 12), employing compensation with the potentiometer, or with both photocells (L and L_c). The galvanometer used by Norberg was a Zernike C (*Kipp and Zoonen*) instrument having a tension-sensitivity of 10,000 scale divisions per volt and a stability level of 3×10^{-15} amp. By means of the Ayrton shunt (V) (Fig. 13) the sensitivity can be reduced by 0.1 and 0.01. The galvanometer is shown at N; the potentiometer for compensation where only one photocell is used at U; R_C resistance $= 1.2 \times 10^{11}$ ohms; R_Q leakage resistance $= 1.1 \times 10^{10}$ ohms. Other resistances are marked in ohms. The apparatus must be mounted within a Faraday cage to avoid electrostatic disturbances and unsoldered contacts must have large frictional contact surfaces.

Manipulations

The filament current in the amplifier is turned on 30 min. before measurements are made, and the tungsten band lamp is also turned on long enough in advance to attain steady illumination. The sample slide on the microscope stage is adjusted so that the image of the exit slit of the monchromator appears in the middle of the sample

drop. The size of the image is 0.12 × 0.12 mm. The slit image is projected over the opening of the measuring photocell (*L*, Fig. 12) by the objective and the prism (*I*). The sector is started; the shutter in front of the photocell is opened; and the photocurrent is compensated by a potential applied by means of the potentiometer (*U*, Fig. 13) to the grid connected to the other photocell so that the zero reading on the galvanometer may be obtained. The galvanometer is usually constant within 0.5 mm. after 1–2 min. The sample slide is now shifted so that the light will pass through solvent alone or a suitable blank. The sector is adjusted until the galvanometer zero reading is again obtained. It is well to repeat the measurements several times.

When the mercury lamp is used as the source of radiation, the photocurrent from the measuring photocell is compensated by the photocurrent from the other cell which is illuminated by the semi-reflecting glass as previously mentioned. Thus, with the mercury lamp the compensation current from the potentiometer is replaced by the compensating photocurrent.

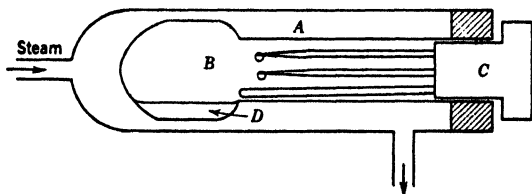
The Sample Slide for Absorption Measurements

Very clean microscope slides and cover glasses are coated with hydrophobic films of nitrocellulose in order to prevent the aqueous drops from spreading. This is accomplished by pouring over the glass a solution of 1 g. of highly nitrated cellulose (about 13% nitrogen), 0.1 g. diethyl phthalate, and 0.01 g. butyl stearate in 100 ml. butyl acetate. The glasses are set aside in a tilted position to drain and dry for at least 3 days in a dust-free place. A drying drum with an electric fan may be used to reduce the drying time. If plastic plates are substituted for the glass, no film is required, but care must be taken that the plastic is optically homogeneous. Two narrow strips of polished glass having a thickness of about 0.35 mm. (from a hemocytometer cover glass) are placed on the hydrophobic film parallel to one another. Between them, sample and blank drops of the order of 0.5–1.0 μl. are pipetted, and paraffin oil is added to fill the area between the glass strips. A cover glass is set on the glass strips to complete the cuvette. To determine the layer thickness in the cuvette:

1. Draw two thin parallel lines 1 cm. apart on the upper surface of the slide with India ink.
2. In a similar manner, draw two lines 3–5 mm. apart on the under surface of the cover glass.
3. Place the cover glass so that the lines on the two glass surfaces intersect.
4. Measure the distance between the upper and lower lines at the four points of intersection by focusing on the lower line with the microscope and then using the micrometer screw to focus on the upper line.
5. Multiply the distance obtained with the micrometer screw by the refractive index of the paraffin oil to get the thickness of the layer.

Accessories for Norberg Technique

Quartz or Supremax Glass (Schott, Jena) Needles. For the isolation and incineration of the sample, fine needles with slightly thickened points are used. The needles are cleaned by boiling in 2 *N* nitric acid and rinsing with distilled water.

Fig. 14. Apparatus for microhydrolysis: *A*, Steam mantle; *B*, chamber of hydrolysis; *C*, holder with quartz needles; *D*, water. *From Norberg (1942)*

Hydrolysis Chamber. The arrangement shown in Figure 14 is used for the hydrolysis of certain constituents in the ash after the sample has been incinerated on the tip of a quartz needle. For the hydrolysis of pyro- and metaphosphate to orthophosphate, Norberg placed 1–2 μl. of 1 *N* hydrochloric acid on the tip of each needle and heated for 1 hr. at 100° in the chamber. Should the acid evaporate in the chamber, the hydrolysis must be repeated. After hydrolysis the drops are allowed to evaporate to dryness at room temperature.

Muffle Furnace. An ordinary muffle furnace may be used for the ashing of the sample on the tip of a quartz needle.

Methods

Phosphorus

By means of his microscopic photometric technique (page 120) Norberg (1942) developed a method for the estimation of phosphorus in quantities down to 0.5 mμg. with an error not greater than about 20% for single analyses. Naturally the error is less with larger samples and greater accuracy is obtained by averaging the results of several determinations. For amounts of phosphorus under 1 mμg., Denigès' stannous chloride method is recommended, while for 1 mμg. and more it is preferable to employ Fiske and Subbarow's aminonaphtholsulfonic acid method, which is technically easier.

Norberg Method for Phosphorus

SPECIAL REAGENTS

0.005 N Calcium Acetate.

1 N Hydrochloric Acid.

Denigès Reagents. (*a*) 0.01 *M* sodium molybdate in 0.6 *N* sulfuric acid; (*b*) 0.2 *N* stannous chloride in concentrated hydrochloric acid, approximately 2.5% $SnCl_2.H_2O$.

Fiske and Subbarow Reagents. (*a*) 0.022 *M* sodium or ammonium molybdate in 0.75 *N* sulfuric acid; (*b*) dissolve 12 g. sodium metabisulfite in 80 ml. water, stir in 0.2 g. 1,2,4-aminonaphtholsulfonic acid (some commercial preparations of this compound are not suitable, that of *British Drug Houses Ltd.* proved to be good) and add 2 ml. 20% crystallized sodium sulfite. Let stand overnight and filter off the undissolved aminonaptholsulfonic acid. Store in a dark bottle.

PROCEDURE

1. Obtain the sample on the tip of a quartz needle (page 123). If the sample is liquid, pipette 1 μl. 0.005 *N* calcium acetate onto the needle tip with the sample and allow to dry in the air. If the sample is solid, the calcium acetate is placed on the tip and allowed to dry before taking on the sample. The excess calcium is required to prevent loss of phosphorus during the incineration.

2. Place the needle in a cold muffle furnace and turn on the heat. When the temperature reaches 500° turn off the heat and remove the needle when the furnace is cool. As an alternative, place the needle in the furnace at 500° and remove it after 20–30 min. at this temperature.

3. Hydrolyze the pyro- and metaphosphate to orthophosphate by pipetting 1–2 μl. 1 *N* hydrochloric acid onto the needle tip and heating for 1 hr. at 100° in the hydrolysis chamber (page 123). Allow to dry at room temperature.

4. For the Denigès method, add 0.05 ml. of the stannous chloride soln. to 10 ml. of the sodium molybdate soln. This mixture must be used within 3 min. after its preparation. Pipette a 0.6 to 1.0 μl. drop of the reagent mixture on a prepared slide near one of the inked lines (page 123) as a blank. Then pipette a suitable vol. (0.6–1.0 μl.) of the reagent mixture onto the needle tip bearing the ashed sample. Use the end of the pipette to mix the drop on the needle tip for 10–15 sec. in order to dissolve the sample and obtain a homogeneous soln. During the next 15 sec., transfer the drop from the needle to the slide, placing it beside the other inked line a few mm. from the blank drop. Surround the drops with paraffin oil and place cover glass as described on page 122. The entire process from the addition of the reagent to placing the cover glass can be performed in 35–45 sec. Standardize the manipulations to maintain a constant evaporation effect. Carry out the photometry after 5 min. from the beginning of the color development, and finish within 45 min.

5. For the Fiske and Subbarow method, mix 24 ml. of the molybdate soln. with 1 ml. of the aminonaphtholsulfonic acid soln. This mixture may be used for at least 1 hr. after its preparation, and it is applied in the same manner as the Denigès reagent. Let the drop stand for 30 min. before starting the photometry, and finish the measurement within 2 hr. from the beginning of the color development.

3. Stowell Technique

The apparatus consists of a lamp, a microscope, a photocell, and amplification and recording equipment. A 50 c.p. automobile headlamp operated by a storage battery is used as the source of light.

The lamp is housed in a Spencer (No. 367) lamp case which has a filter holder. A microscope fitted with a mechanical stage, a 44 × achromatic objective, and a 15× compensating ocular are employed. The field of observation is limited to an area 50 × 35 μ by inserting a rectangular diaphragm in the ocular. The light from the microscope is thrown on a vacuum photocell (RCA 929) enclosed in a light-tight box having a side tube to extend over the microscope tube. This side tube contains a movable mirror so mounted that it can be interposed in the light path to enable inspection of the field, and then turned out of the path to permit photoelectric measurement. The photocell current is amplified by a General Electric FP54 tube in a Barth circuit with a 10^{10} ohm grid resistor (Penick, 1935). The amplified current is measured with a Leeds and Northrup student type potentiometer and a Leeds and Northrup type R galvanometer.

Measurements are made of the light transmittance through both stained and unstained sections in order to obtain the absorption due to the stain itself. In both cases, measurements are also made of the transmittance through blank portions of the glass slides adjacent to the sections to correct for variations in the intensity of the light source, changes in amplification or potentiometer batteries, and alterations in thickness of cover glasses, slides, or mounting media. When it is possible, fifty adjacent areas on each section are measured and the mean percent absorption calculated.

Stowell and Albers (1943) employed a Coleman Model 10 S double monochromator spectrophotometer by means of which they measured absorptions of light bands, having a 5 mμ spectral width, by stained sections of tissue. Since no microscope is employed in this apparatus, the absorption of the section as a whole, rather than a chosen cellular region, is measured.

The photometric procedure was employed by Stowell (1942) for the estimation of desoxyribonucleic acid in tissue sections by means of the Feulgen stain (page 65). Light from the source was passed through a heat-absorbing filter (Corning Aklo No. 396) and a green gelatin filter (Wratten No. 58) before entering the microscope. The extension of the method to absorption studies with a variety of stains commonly used in histological examination was included in the reports of Stowell and Albers (1943) and Stowell (1945b). Subsequently Stowell (1945a) subjected the Feulgen reaction to detailed study and described each step in his method of using it.

D. ROENTGEN ABSORPTION HISTOSPECTROSCOPY*

One of the most significant advances in histo- and cytochemical technique has come from the work of Engström (1946) at Karolinska Institutet, Stockholm, who, by employing the roentgen absorption of tissue sections or of very small volumes of liquid, developed a procedure whereby quantitative elementary analyses can be directly performed with an accuracy of about 5–10% on 1×10^{-9} to 1×10^{-12} gram of material, *i.e.*, quantities of the order found in single mammalian cells. Thus, in specific instances, phosphorus and calcium can be determined in a 10 μ section of bony tissue within an area around 10 × 10 μ, and nitrogen and oxygen in a section a couple of microns thick within an area of 50–100 μ^2. A particular advantage of this technique is that the tissue is not used up, and hence may be subsequently employed for histological study by the usual methods so that direct correlation may be made between the chemical composition and the microanatomical structure. Furthermore, the analysis is independent of the chemical structure in which the element may be bound, and the physical state of the specimen is unimportant, *e.g.*, fixed tissue, dry powder, and in certain cases, paraffin-embedded tissue, or solutions, may be used. A number of elements can be determined on the same sample.

The advantages of being able to determine the total quantity of a tissue element *in situ* without regard to its chemical form and state of valence, or affiliations with other elements, are hardly to be minimized. The method is confined to the quantitative determination of elements having an atomic number of 6 (carbon) or greater. This would include all elements of biological importance with the exception of hydrogen.

Engström (1946) has pointed out that roentgen spectroscopic methods based on emission analysis are not well suited for elements with atomic numbers less than 20, and the emission methods cannot be applied very well to the small surfaces involved in histo- and cytochemical studies. This applies to the earlier procedure of von Hamos and Engström (1944) in which tissue is subjected to roentgen radiation and the secondary radiation is measured for the quantitation of constituent elements.

* See Bibliography Appendix, Refs. 23, 24, 25, 26, and 27.

The use of the roentgen absorption method requires an understanding of the theoretical basis of roentgen spectroscopy. All that can be included here are a few of the more salient features of the theoretical treatment which Engström (1946, pages 19–50) applied to his method, a description of the apparatus, and consideration of certain other practical aspects. This information can serve merely to acquaint the reader with the method, the actual use of which will depend on the understanding of roentgen spectroscopy mentioned previously and a detailed study of the presentation of Engström (1946).

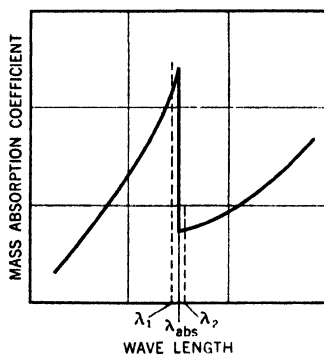



Fig. 15. Schematic representation of an absorption jump. *From Engström (1946)*

1. Some Theoretical Aspects

Quantitative analysis based on the roentgen absorption utilizes the absorption discontinuities which appear at a characteristic wavelength for every element. The absorption of an element near the K-absorption edge is indicated in Figure 15. The determination of the element depends on the measurement of the absorption of monochromatic radiation with wavelengths on each side of, and close to, the absorption edge of the element to be determined.

The mass (x), in g./cm.², of the element to be analyzed is given by the following equation:

$$x = \frac{\ln \frac{i_2}{I_2}\left(\frac{\lambda_1}{\lambda_2}\right)^P - \ln \frac{i_1}{I_1}}{\frac{\mu_1}{\rho} - \frac{\mu_2}{\rho}\left(\frac{\lambda_1}{\lambda_2}\right)^P}.$$

TABLE III

Wavelength of the Absorption Edges for Certain Elements and Suitable Analysis Lines (Engström, 1946)

Element	Atomic number	K-absorption edge, X.U.	λ_1 Line	λ_1 Wave-length, X.U.	λ_2 Line	λ_2 Wave-length, X.U.
C	6	43700	20 Ca $K\alpha$	36270	6 C $K\alpha$	44540
N	7	31000	22 Ti $L\alpha$	27370	21 Sc $L\alpha$	31370
O	8	23300	24 Cr $L\alpha$	21530	23 V $L\alpha$	24310
Na	11	~11500	32 Ge $L\alpha_1$	10415	11 Na $K\alpha_1$	11885
Mg	12	9496	34 Se $L\alpha_1$	8972	33 As $L\alpha_1$	9652
P	15	5775	41 Nb $L\alpha_1$	5712	40 Zr $L\alpha_1$	6057
S	16	5009	44 Ru $L\alpha_1$	4836	16 S $K\alpha_1$	5361
Cl	17	4384	46 Pd $L\alpha_1$	4359	45 Rh $L\alpha_1$	4588
K	19	3431	20 Ca $K\alpha_1$	3352	51 Sb $L\alpha_1$	3432
Ca	20	3063	21 Sc $K\alpha_1$	3025	20 Ca $K\alpha_1$	3352
Fe	26	1740	70 Yb $L\alpha_1$	1668	68 Er $L\alpha_1$	1780
Cu	29	1377	77 Ir $L\alpha_1$	1348	76 Os $L\alpha_1$	1389
Zn	30	1280	79 Au $L\alpha_1$	1274	78 Pt $L\alpha_1$	1310
As	33	1043	81 Tl $L\alpha_1$	1013	79 Au $L\beta_1$	1081
Br	35	918	92 U $L\alpha_1$	909	37 Rb $K\alpha_1$	924
Ag	47	484	51 Sb $K\alpha_1$	469	50 Sn $K\alpha_1$	490
I	53	373	57 La $K\alpha_1$	370	56 Ba $K\alpha_1$	384

Element	Atomic number	L_{III} absorption edge, X.U.	λ_1 Line	λ_1 Wave-length, X.U.	λ_2 Line	λ_2 Wave-length, X.U.
Ca	20	35630	21 Sc $L\alpha$	31370	20 Ca $L\alpha$	36270
Cu	29	13150	30 Zn $L\alpha$	12250	29 Cu $L\alpha$	13600
Ag	47	3691	50 Sn $L\alpha_1$	3592	19 K $K\alpha_1$	3734
I	53	2714	57 La $L\alpha_1$	2660	22 Ti $K\alpha_1$	2743
Hg	80	1008	34 Se $K\beta_1$	990	35 Br $K\alpha_1$	1038
Bi	83	922	92 U $L\alpha_1$	909	37 Rb $K\alpha_1$	924

where (μ_1/ρ) and (μ_2/ρ) represent the mass absorption coefficients for the element at wavelengths λ_1 and λ_2, respectively, ρ, the specific gravity of the absorbing substance, and μ, the linear absorption coefficient; i_1 and i_2 are the intensities of transmitted radiation of wavelengths λ_1 and λ_2, respectively, and I_1 and I_2, the corresponding intensities of the incident radiation. P is a function of atomic number and wavelength. When the wavelengths are selected close to the absorption edge, and the intensities of the incident radiation of both

wavelengths are equal, *i.e.*, $I_1 = I_2$, the equation may be simplified as follows:

$$i_1 = i_2 e^{-(\mu_1/\rho - \mu_2/\rho)x} = i_2 e^{-kx}$$

Values for (i) and (I) are determined experimentally, and (μ/ρ) can be obtained from tables of mass absorption coefficients for different elements and wavelengths, or it may be calculated.

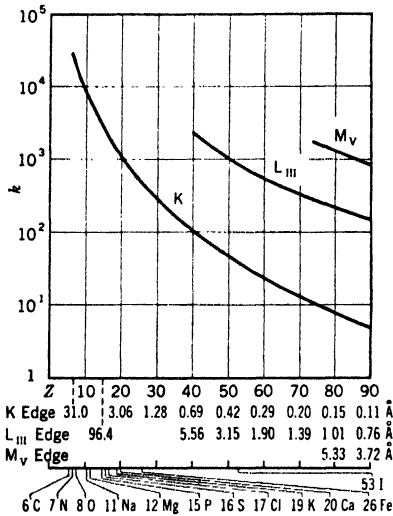



Fig. 16. Value of k, that is, $(\mu_1/\rho) - (\mu_2/\rho)$, for different absorption edges and different elements. For 15 P the wavelength for the L edge is $L_{II} - L_{III}$. *From Engström (1946)*

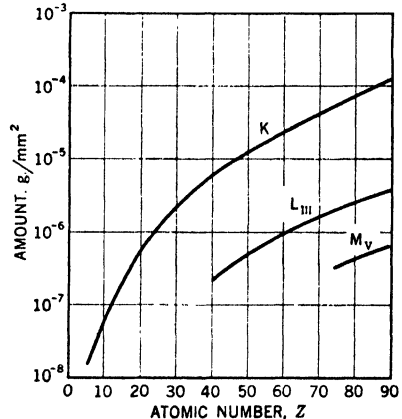



Fig. 17. The smallest determinable amount of an element. *From Engström (1946)*

From the preceding equation it follows that the smallest determinable quantity of the element per unit of surface is a function of k or $(\mu_1/\rho) - (\mu_2/\rho)$. Accordingly, an absorption edge should be chosen at which the difference between the mass absorption coefficients is as large as possible. The variation of k with elements of different atomic numbers (Z) is shown in Figure 16, which illustrates that the K-absorption edge must be used for elements of low atomic number, the L_{III} edge for elements in the middle of the periodic table (the L_{III} is of greater magnitude than L_I or L_{II}), and the M_V edge for the

TABLE IV

Elementary Composition of Muscle (Engström, 1946)

Element	Atomic number	m. $\times 10^9$ g. $\times$ cm.$^{-2}$ $\times$ μ^{-1}	20 Ca, Kα 3353 X.U.		51 Sb, Lα_1 3432 X.U.	
			$\frac{\mu}{\rho}$	m. $\times \frac{\mu}{\rho} \times 10^7$	$\frac{\mu}{\rho}$	m. $\times \frac{\mu}{\rho} \times 10^7$
C	6	11500	50	5750	55	6330
N	7	3600	80	2880	85	3060
O	8	5000	115	5750	120	6000
Na	11	70	265	190	285	200
Mg	12	20	350	70	370	70
P	15	170	635	1080	680	1160
S	16	200	790	1580	840	1680
Cl	17	60	885	530	945	570
K	19	370	1215	4500	125	460
Ca	20	2	145	3	150	3
Fe	26	20	310	60	330	70
				$\Sigma 2.239 \cdot 10^{-8}$		$\Sigma 1.960 \cdot 10^{-8}$

heaviest elements (the M_V is the greatest of M_{I-V}). The smallest quantity of an element, in g./mm.^{2}, which can be determined is given in Figure 17. This is based on the fact that the smallest intensity difference between i_1 and i_2 which can be measured with certainty is about 5%.

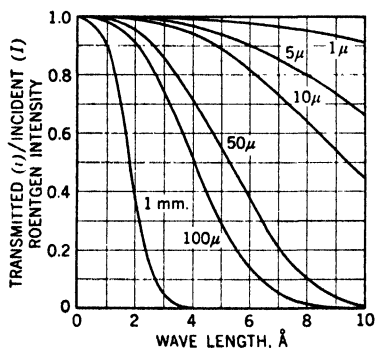



Fig. 18. Absorption of roentgen radiation of different wavelengths in paraffin of varying thickness. *From Engström (1946)*

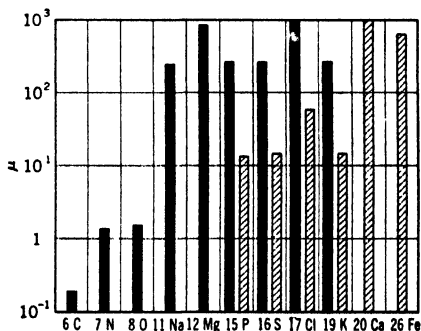



Fig. 19. Appropriate layer thicknesses for determinations of different elements in muscular tissue. The solid columns indicate the layer thickness when the K-absorption edge is used; the hatched, the L_{III} edge. *From Engström (1946)*

2. Thickness of Sections

The tissue to be subjected to elementary analysis must be frozen-dried and infiltrated with paraffin by a procedure such as that of Packer and Scott (page 5). Removal of the paraffin from sections of this tissue would involve extractions and displacements of elements by the action of the solutions required. Therefore, it is preferable to carry out the absorption analysis without removing the paraffin. The absorption of the paraffin itself is demonstrated in Figure 18. The curves were plotted for $C_{25}H_{52}$ (sp. gr. 0.90); they show that, up to 3 Å, the paraffin may be used in a layer up to 50–100 μ, while for 5–6 Å the layer should not exceed 10 μ, etc.

A summary of Engström's calculations of appropriate section thickness for analyses of muscle tissue is given in Figure 19. It is considered prerequisite that the quantity of an element to be analyzed be at least about twice the smallest quantity which can be determined. Fig. 19 was derived from data such as in Table IV,

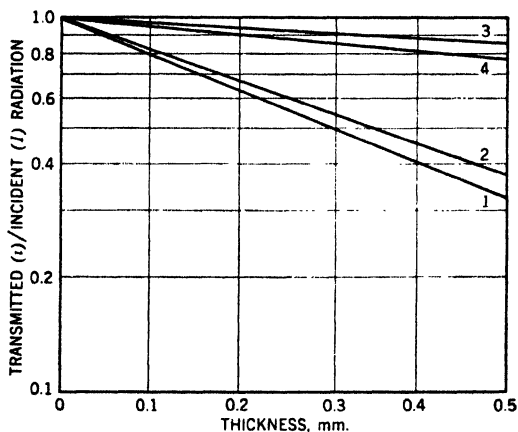



Fig. 20. Analysis of 19 K in muscular tissue: (1) i_1/I_1; (2) i_2/I_2; (3) i_1/i_2 for $I_1 = I_2$; (4) i_1/i_2 for wavelengths very close to the absorption edge and $I_1 = I_2$. See Table IV. *From Engström (1946)*

which gives the elementary composition of muscle and the mass absorption coefficients and absorption capacity of the elements with the wavelengths used. The wavelength of the K-absorption edge for potassium (atomic number 19) is 3427 X.U. (1 X.U. = 0.001 Å), and hence, for the potassium determination analysis lines on each side, 3353 and 3432 X.U. are used in the table. A specific gravity of 1.0 for the tissue has been used in the calculation, and the layer

thickness is 1 μ. The data in the table have been used to obtain the curves in Figure 20, and from these it appears that sections 0.2–0.3 mm. thick are appropriate for potassium analysis. Thus, in a volume of 0.001 μl. of muscle tissue having a surface of 0.01 mm.^{2} and a potassium content of 0.3% the quantity analyzed will be 3×10^{-9} g.

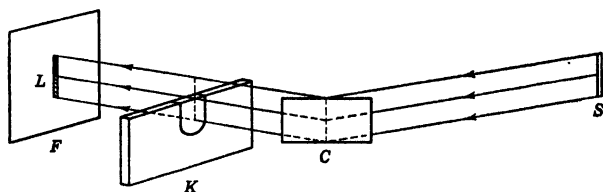



Fig. 21. Schematic picture showing the arrangements for analysis in a microcuvette: S, slit in roentgen tube; C, the crystal; K, the microcuvette, F, the photographic film. In place of K a microscopic section can be used. *From Engström (1946)*

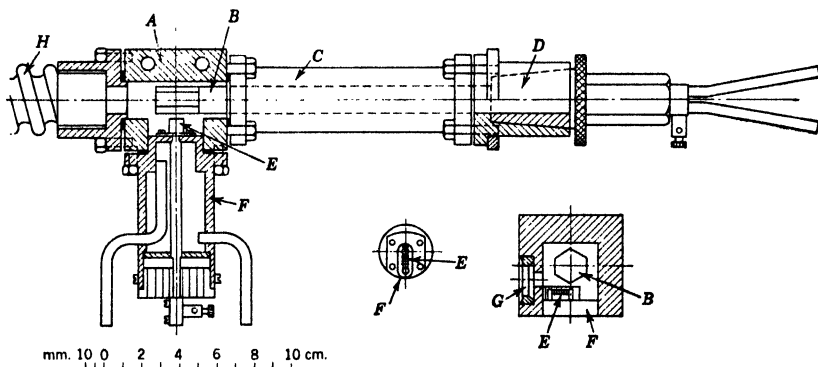



Fig. 22. Roentgen tube for primary excitation.
From Engström (1946)

3. Apparatus

The arrangement shown in Figure 21 enables the simultaneous determination of the incident and transmitted radiation intensity. The former is proportional to the blackening on the upper part of line *L*, and the latter to the lower part. The roentgen tubes employed by Engström to produce the radiation were operated by a direct-current unit manufactured by *G. Schönander Co.*, Stockholm. This

unit was designed to develop 1.5 kilowatts at 50 kilovolts or 4.5 kilowatts at 15 kilovolts. The evacuation of the roentgen tubes was accomplished by a three-stage mercury diffusion pump connected to a two-stage mechanical forepump. A cooled trap was placed between the tubes and the pumps.

Roentgen Tube for Primary Excitation. In order to obtain a line spectrum of great intensity it is best to solder the element whose line spectrum is desired to the anode. This cannot always be done, but for elements which lend themselves to this procedure, the anode is made with six surfaces having a different element or suitable alloy of it on each surface. A diagram of the tube is shown in Figure 22. The forged brass body (*A*) has four openings and bored channels for cooling. The water-cooled cathode (*F*) with filament *E* is sepa-

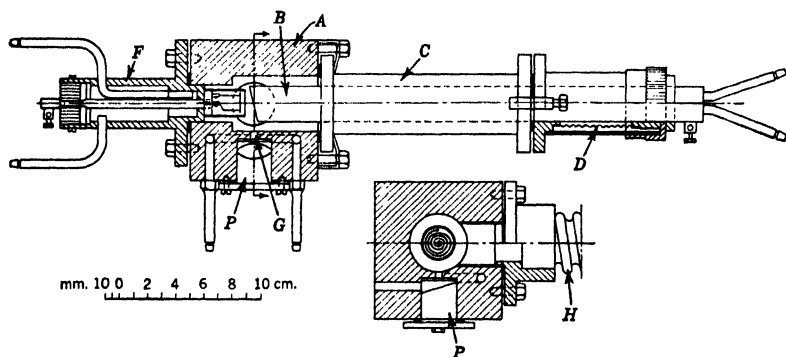



Fig. 23. Roentgen tube for secondary excitation.
From Engström (1946)

rated from *A* by rubber packing. The anode (*B*) is insulated from *A*, which is grounded, by the porcelain tube *C*. A rubber packing separates the cone (*D*) from *C*. The anode may be turned to present its different surfaces to the cathode without breaking the vacuum by virtue of an Apiezon grease packing between it and *D*. The anode is water-cooled. The roentgen beam is passed through slit *G* which is covered with an aluminum foil (9 μ thick) fastened on with Apiezon grease. The flexible tube *H* connects to the vacuum apparatus. The filament (*E*) consists of a 0.25 mm. platinum wire winding which is coated with an oxide layer (made by burning off sealing wax).

Roentgen Tube for Secondary Excitation. When the element whose line radiation is desired cannot be soldered to the anode, the oxide or the powdered metal must be used. However, the difficulties of suitably incorporating these powders in the surface of the anode led Engström to the use of secondary excitation outside of the high vacuum. The resulting reduction in intensity was compensated in some measure by the use of greater wattage and by obviating the passage of the radiation through a window or membrane.

The roentgen tube is illustrated diagrammatically in Figure 23. The brass body (*A*) is 90 × 90 × 100 mm. The cathode filament consists of a flat platinum spiral with an oxide coating. An iron cylinder surrounds the filament to direct the electron stream. The tube is evacuated through *H*, which is 42 mm. in diameter. The surface of the anode (*B*) forms a 12° angle, and the porcelain tube

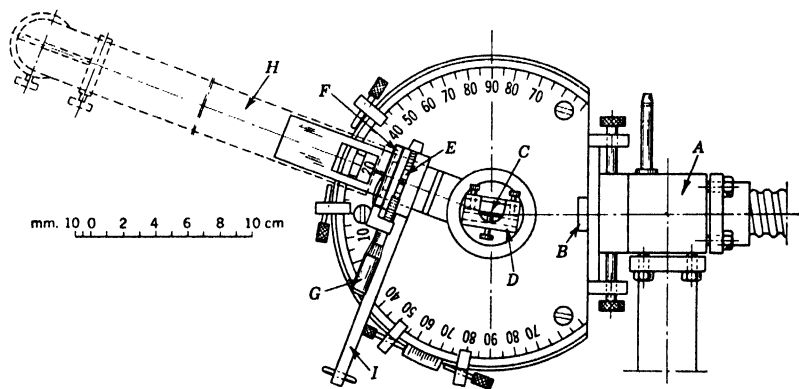



Fig. 24. Spectrograph for microanalysis.
From Engström (1946)

C insulates the anode. Adjustment of the anode position under vacuum is obtained by a bellows arrangement (*D*). Water-cooling is employed for the body of the tube, the cathode, anode, and slit *G*. An aluminum foil window (9 μ thick) is placed over the slit, and a plug (*P*) is used to hold the element whose line radiation is desired. This plug and all of the vacuum connections are sealed with rubber. The composition of the anode is chosen to give the greatest yield of secondary radiation from *P*. Hevesy has shown that the greatest yield of characteristic radiation results when the incident line radi-

ation has a wavelength 200–600 X.U. shorter than that for the absorption edge of the element whose secondary radiation is desired. Accordingly, it is preferable to use K-radiation from copper (atomic number, 29) to excite K-radiation from iron (26). If there are no suitable lines for the secondary radiation the continuous radiation from an element of high atomic number such as tungsten (74) or platinum (78) is used.

Spectrograph for Microanalysis. For wavelengths shorter than 2.5 Å it is not necessary to enclose the spectrograph in a vaccum, since the absorption due to air becomes appreciable only for wavelengths greater than 2.5 Å. The spectrograph for microanalysis is

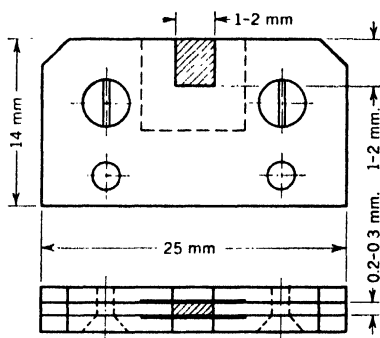



Fig. 25. Microcuvette. The hatched area is the volume employed. *From Engström (1946)*

used with these shorter wavelengths; the diagram of the instrument is shown in Figure 24. Either photographic or ionization measurements may be made of the radiation intensity with this apparatus. The roentgen tube (*A*) delivers a stream of radiation through the spectrograph slit (*B*) to the crystal (*C*) which is mounted in the holder (*D*). The monochromatic beam produced passes both above and through the microcuvette (*E*) and onto the photographic film in the holder (*F*). The cuvette can be moved relative to the film by the micrometer screw (*G*). An ionization chamber (*H*) may be substituted for the photographic film; the chamber is directly connected to an electrometer by its central electrode. (An Edelmann string electrometer was employed.) A Bakelite bar (*I*) operates a cog by which the cuvette may be moved into or out of the path of

the beam when the ionization chamber is used. Adjustments on the circular scale can be made to some hundredths of a degree, and stops on the scale can be set to enable rapid and accurate changes for different wavelengths.

The construction of the metal microcuvette is shown in Figure 25. The walls in the direction of the radiation are made of thin aluminum foil, glass, or cellophane sheets. The cuvette capacity is 0.2 μl. or greater. Crystals of calcite (d = 3029 X.U.) and rock salt (d = 2814 X.U.) were used and the ionization chamber was charged to 150 volts.

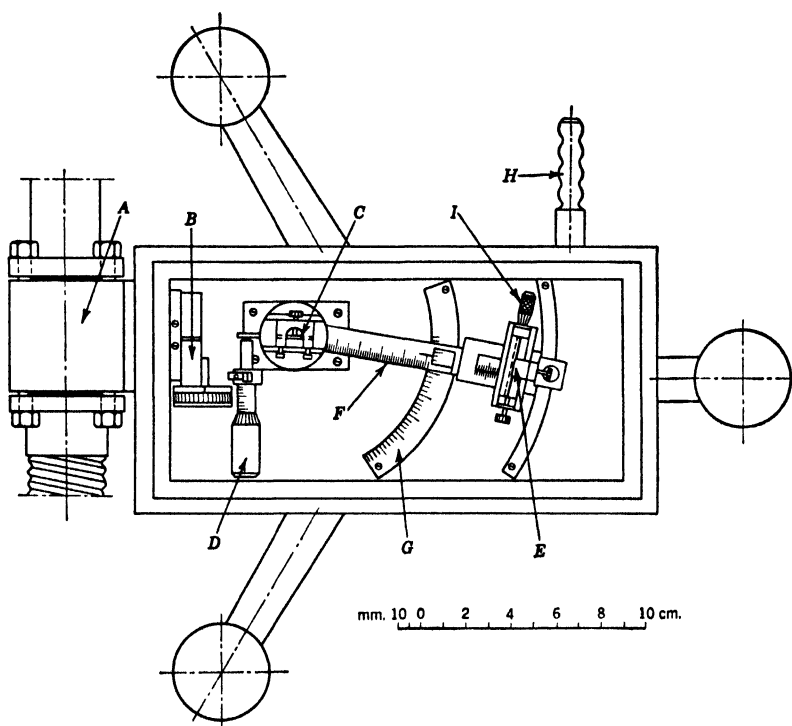



Fig. 26. Vacuum spectrograph for histochemical analysis.
From Engström (1946)

Vacuum Spectrograph for Tissue Analysis. The construction of the vacuum spectrograph is shown in Figure 26. The roentgen tube (A) is joined to the spectrograph by an air-tight rubber gasket.

The radiation enters through the adjustable slit (*B*) and falls on the crystal (*C*), whose angle may be adjusted by the micrometer screw (*D*). The monochromatized beam from the crystal passes through the tissue section in holder *E*. The holder can be moved in relation to the photographic film placed behind the tissue by means of the micrometer screw (*I*). The film carriage can be adjusted along scale *F* at a chosen distance from *C*. The angle of the tissue holder can be set on scale *G*. The spectrograph whose dimensions are $10 \times 20 \times 6$ cm. is evacuated through *H*. The lid is sealed to the chamber with rubber and clamped tight. The films used were 12×12 mm. and the crystals employed were gypsum ($d = 7578$

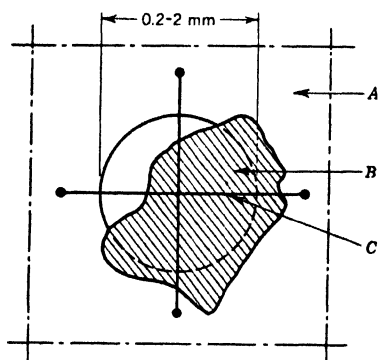



Fig. 27. Mounting of a preparation. *A* is a part of the preparation holder. *B* is the preparation itself. *C* is a cross of Wollaston wires (platinum) used to obtain points of reference.

From Engström (1946)

X.U.) and mica ($d = 9930$ X.U.). The tissue section is mounted over a hole (0.2–2 mm.) in a sheet of metal, Figure 27. The distance between tissue and film is 1.5–2 mm.

4. Measurement of Density of Photographic Film

The intensity of the radiation is measured by the degree of the blackening of the photographic film. The blackening may be determined by photometric estimation of the proportion of visible light absorbed by the film or by measurement of the proportion of the silver that has been reduced.

The photometric estimation has been effected by two methods in Engström's (1946, pages 60–64) work. One procedure utilized a self-recording microphotometer (Siegbahn type) with a thermoelement and Moll's microgalvanometer, and the other employed the Caspersson photoelectric apparatus for the determination of light

absorption in very small areas. The latter method was previously described in connection with Caspersson's absorption technique for cells (page 114) and Norberg's technique for fluids (page 120).

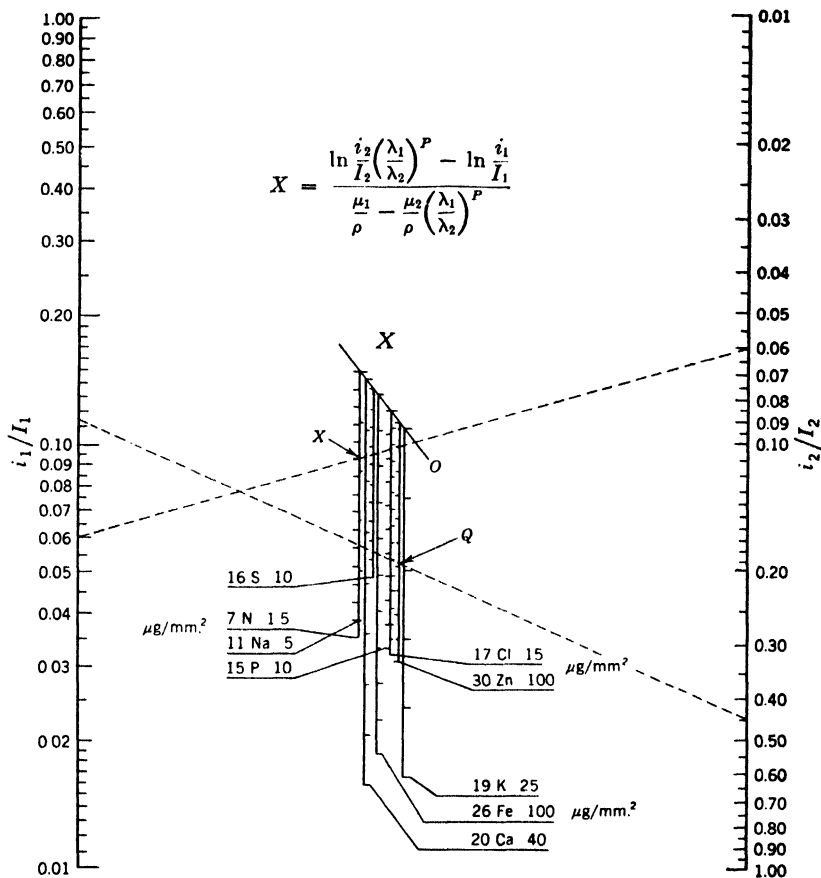



Fig. 28. Nomogram for calculating analytical results by equation shown. *From Engström (1946)*

The light absorption in areas less than 1 μ^2 can be accurately measured with this apparatus; however, the size of the grains, even in the finest films, makes it necessary to measure the blackening in areas 10×10 μ.

The method for the estimation of the proportion of reduced silver is based on counting the silver grains in the photographic emulsion on the film, following the procedure of Günther and Wilcke (1926). The method is only adapted to low densities (upper limit d = 0.27). A microscopic enlargement of 600× is used for coarse-grained film and 1350× (immersion objective) for fine-grained film. The counts are facilitated by the use of a netted ocular having 100 squares. The number of grains in an unexposed film area is determined in a region immediately adjacent to that exposed.

In a study of the properties of photographic emulsions, Engström (1946, pages 65–72) pointed out that it is necessary to obtain a curve of the relationship of photographic density to radiation intensity for every wavelength, emulsion, and set of development conditions in order to arrive at a suitable working arrangement. Engström investigated the properties of Agfa, Laue, and Printon films and Ilford High Resolution plates and presented curves of both density and number of grains as functions of intensity.

Nomogram

Engström (1946) has published a nomogram for calculating the analytical results according to the equation shown in Figure 28:

"In this nomogram are included the most important elements, and the analysis assumes the employment of the K-absorption edge. The wavelengths for the analysis lines used are seen [Table III]. The two outer pillars in the nomogram indicate the quotient between the intensity of the transmitted and incident roentgen radiation in the two wavelengths λ_1 and λ_2. The amount sought for (X) of the respective elements is marked out on the vertical lines in the centre. The figures after the respective elements indicate the amount of the element in question at the end of the scale, *e.g.*, 30 Zn 100 μg./mm.^{2} The following example shows how it is employed: In determinations of nitrogen, it is, *e.g.*, found that i_1/I_1 is 0.06 and that i_2/I_2 is also 0.06. The straight line which joins these two points on the outer pillars cuts the curve for nitrogen at the point X. The end point on the nitrogen scale is 1.5 μg.N/mm.^{2}, and the scale is divided up into 15 parts, from which it appears that the amount of nitrogen sought for is 0.49 μg./mm.^{2}"

E. MICROINCINERATION

Microincineration is a valuable technique for the faithful representation of the total mineral distribution in tissue sections. In its present state of development, its reliability is evidenced by the fact

that motion pictures of incinerating sections of skeletal muscle and of anterior horn cells at 700–800× magnification reveal no distortion, Scott (1943). The advantage of microincineration over chemical tests for the determination of the anatomical disposition of mineral constituents lies in avoiding inevitable displacements and losses resulting from the use of solutions. In addition, the danger of fortuitous adsorption of reagents on colloidal protoplasmic surfaces is circumvented. The present limitations of the technique lie in its essential morphological character, which leaves not only quantitative but qualitative chemical considerations very largely in the dark. Only a few elements can be detected in incinerated preparations, and only a rough estimate of the quantity of ash in a given location can be made.

The microincineration technique was originally developed by Policard and co-workers in France and was introduced in America by Scott. The more recent refinements have resulted chiefly from the careful and extensive researches of Scott and collaborators. The earlier reviews by Policard (1931–1932) and Policard and Okkels (1931) and the later ones by Scott (1933a,1937,1943) and Gage (1938) thoroughly cover the development and applications of this technique.

Engström (1944) carried out a very nice study on the localization of mineral salts in striated muscle fibers by employing ultraviolet absorption followed by microincineration of the same section. By correlation of both techniques he was able to conclude that the intensely absorbing isotropic segments which contain the adenylic acids yielded the ash, whereas practically no ash was derived from the weakly absorbing anisotropic segments.

1. Preparation for Incineration

Some of the earlier work dealt with the use of various solutions for the fixation of tissue in preparation for microincineration, and it was found that absolute alcohol or an alcohol-formalin mixture was best since their use resulted in a smaller loss of mineral matter than was observed with other fixatives. In Scott's (1937) hands the intracellular distribution of minerals was preserved remarkably well when the tissue was fixed for 24 hr. in a solution of 9 vol. absolute alcohol and 1 vol. neutral formalin followed by treatment

with absolute alcohol, clearing in xylol, and embedding in paraffin in the usual fashion. But Scott was quick to point out that the advantages of freezing-drying the tissue are particularly important in studies of this nature and hence freezing-drying is the procedure of choice.

Paraffin sections, 3–5 μ thick, yield the most satisfactory cytological details. The sections are placed directly on ordinary glass slides of good quality and no adhesive is required to make them adhere to the glass. While the presence of water is scrupulously avoided, a drop of absolute alcohol or liquid petrolatum may be employed to flatten the sections if necessary (Policard and Okkels, 1930). If alcohol is used, it is allowed to dry; if petrolatum, it is drained from the slide before the sections are incinerated. The greatest care must be exercised at all times to avoid contamination with dust. Absolutely clean paraffin must be used; and the slides should be washed in distilled water repeatedly, rinsed with filtered alcohol, dried with a clean lint-free cloth, and stored in a dustproof container.

It is good practice to cut serial sections using alternate ones for incineration and the others for controls to be stained and mounted in the usual manner. Scott (1937) pointed out that the use of the cold knife for sectioning, as recommended by Schultz-Brauns (1931), is undesirable since condensation of moisture on its surface may result in some wetting of the tissue.

2. The Incineration Furnace

The furnace used for the incineration of tissue sections is simply a quartz tube electrically heated by windings of resistance wire. Ordinary laboratory muffle furnaces can be used if their temperatures can be properly regulated and if sufficient care is taken to protect the slides from possible contamination inside the furnace.

Scott (1937) constructed a very convenient furnace capable of uniform and reproducible performance. It consists of a quartz tube 24 in. long that is wound with three separate 600 watt heating units and the whole covered with asbestos insulation. Each heating unit is controlled by a 44 ohm, 3.2 amp. rheostat. The slides are slowly moved through the furnace tube on quartz slabs by means of an electric motor operating through a speed-reducing worm gear

system. A rack running through the tube and extending from both ends serves to support the quartz slabs as they are moved along. Further details of the apparatus have not appeared.

3. Scott Incineration Procedure

Scott (1937) gave the following directions for incineration:

1. Gradually bring to 200° over 10 min.
2. Gradually elevate to 280° over the next 5 min.
3. Gradually elevate to 385° over the next 5 min.
4. Gradually elevate to 480° over the next 5 min.
5. Gradually elevate to 580° over the next 5 min.
6. Gradually elevate to 650° over the next 3–5 min.
7. Shut off furnace and let cool for 5–10 min.
8. Remove slides from furnace and place cover slip over section as soon as cool enough to handle. Seal edges with a mixture of 1 part paraffin, 1 part beeswax, and 1 part resin (by weight). The use of a cover slip permits observation with an oil immersion objective, and it prevents absorption of moisture and efflorescence of the ash. Greatest care is advised to avoid any air current between the time of removal from the furnace and sealing the cover slip, since the ash is easily disarranged.

The practice of covering the ash with collodion or Canada balsam is undesirable, since it involves the danger of disarrangement and disturbance of optical properties.

Variation in the above procedure may be necessary for particular tissues. The greatest shrinkage occurs between 60–70°, and especially in tissues rich in elastic and fibrous material such as blood vessels. In order to produce practically all of the shrinkage in advance, Policard and Ravaut (1927) place fixed tissues in absolute alcohol and bring slowly to the boiling point. However, this procedure is not advised by Scott for cytological studies because there is the possibility of dissolving salts.

The passage of a stream of nitrogen through the tube during incineration was recommended by Schultz-Brauns (1929), and Tschopp (1929) suggested a similar use of oxygen. Policard (1933b) employed nitrogen containing a small concentration of oxygen, which he claimed effects more rapid oxidation. Although satisfactory results have been obtained with these methods, and the use of oxy-

gen seems to permit lower incineration temperatures which result in less volatilization of chlorides, nevertheless it is sufficient as a rule to carry out the treatment in air.

4. Microscopic Examination and Interpretation

Observations of incinerated sections should be made under the microscope with dark-field illumination provided by a cardioid condenser. A proper light source is an important factor and Scott (1937) pointed out that a carbon arc seems to produce excessive longitudinal aberration, while a Spencer illuminator fitted with a 500 watt projection lamp or a Zeiss Point-O-Light lamp, with proper centering of the condenser and adjustment of the mirror, are suitable. Unscreened light is best for observation of minute particles, but the use of a daylight filter or ground glass is more restful to the eye. Mercury vapor lamps enable high resolution of small particles but make recognition of colors difficult.

The use of a comparing ocular with two microscopes is recommended for simultaneous observation of both an incinerated section and its stained control. Of course, the stained section is illuminated in the ordinary bright field.

If the incineration has been carried out properly, there will be no black or brownish carbon deposits. The topographic disposition of the ash no doubt fairly represents the distribution of mineral constituents in the fixed tissue. That this distribution is exactly maintained in the living tissue cannot be said with certainty; however, Scott (1932) has found the parallelism that histological sites in living tissues that absorb ultraviolet radiation (275 mμ) are those which yield large amounts of ash on incineration.

The only elements that can be identified in the ashed sections with any measure of certainty are considered in the following sections.

Sodium and Potassium. It has been assumed that sodium and potassium yield a fine-grained, faintly bluish-white ash. Policard and Pillet (1926) attempted the identification of sodium and potassium as the sulfates by exposing sections, before incineration, to the fumes of sulfuric anhydride in order to convert the chlorides to sulfates. The sulfates are resistant to volatilization during the ashing, while the chlorides are apt to be lost.

Calcium and Magnesium. The dense white ash seen in the dark field is due chiefly to calcium with a smaller amount of magnesium. Unless spectrographic means are employed, magnesium cannot be identified in the presence of calcium in incinerated sections. As a test for calcium, Moreau (1931) suggested dissolving ash in a "microdrop" of 0.1 *N* hydrochloric acid followed by the addition of a "microdrop" of 0.1 *N* sulfuric acid in order to form the needle-shaped crystals of calcium sulfate.

Silicon. The identification of silicon can be made with assurance since silica retains its typical crystalline structure during incineration, and its double refraction when examined with polarized light serves as an additional means of characterization (Policard and Martin, 1933). The tendency of certain constituents in the ash to combine with the silica in the glass slide during the heating may give rise to a misleading appearance. The probability that silica and calcium salts combine in the incineration process must also be kept in mind.

Iron. The oxidation of iron that occurs produces a color in the ash which may vary from yellow to deep red making the identification of this element relatively simple. Scott (1937) cautions that care must be taken to avoid contamination with iron from the microtome knife. A newly sharpened knife will be apt to cause the most trouble. After 40–50 sections have been cut the number of particles of iron left in the subsequent sections is practically negligible.

Lead. Exposing incinerated sections to hydrogen sulfide gas has been employed by Tada (1926) and Okkels (1927) for the identification of lead as its black sulfide. However, sulfides of other metals are also black and the possibility of an interference of this nature should be kept in mind. It is necessary to make sure that carbon particles due to faulty incineration are not present before the ash is subjected to the gas, since these particles and the black sulfide can be easily confused.

Uranium. Policard and Okkels (1930) claimed to have detected uranium, by its fluorescence under ultraviolet radiation, in ashed sections from animals poisoned by this element. Fluorescences may be produced by impurities too, and hence this criterion is not a very rigorous one.

5. Quantitative Estimation of Ash

Attempts at the quantitation of the relative amounts of ash left by various structures were made by Schultz-Brauns (1931) based on a standardized development of photomicrographs. However, this method has many inherent difficulties and can yield little.

Scott (1933b), and Williams and Scott (1935) developed a photoelectric apparatus to measure the intensity of light reflected from the ash. This light intensity is roughly proportional to, and serves as an approximation of, the quantity of the mineral residue. While the method obviously leaves much to be desired, as Scott would no doubt be the first to admit, it is capable of furnishing some information that at present can be obtained in no other way.

An idea of the sensitivity of the apparatus, as assembled and used by Williams and Scott, may be gained from the fact that, with a magnification of 700×, the ash produced in a 5 μ section by a single hepatic cell nucleus results in a galvanometer deflection of about half-scale (25 cm.).

Williams and Scott Photoelectric Apparatus. The microscope illumination is furnished by a 6 volt, 108 watt ribbon filament projection lamp enclosed in a ventilated housing. This lamp is supplied through a constant-voltage regulator and a step-down transformer. The slide is held by a special mechanical stage which has rack and pinion adjustment laterally and vertically and a fine screw adjustment axially; and the microscope, mounted horizontally, is fitted with a Zeiss aplanatic 1.2 condenser, a Leitz No. 3 objective, various oculars, and a clamped-on 90° reflecting prism. A fixed diaphragm made of a disc slightly larger than the aperture of the objective is fitted into the ring on the condenser lens. The light emerging from the ocular is reflected by the prism to a mirror which in turn reflects it to the photocell.

The gas-filled photocell is mounted in a light-tight copper box which serves as an electrostatic shield as well. The cover of this box carries rotatable and interchangeable 3 in. white cardboard discs with various size openings to determine the illuminated area on the photocell. A shutter under the disc permits exposure of the photocell when desired. Within the copper box containing the photocell, a FD54 Pliotron tube is mounted with its 10^9 ohm high-resistance shunt. The photocell is connected directly to this tube, and the

connections to the DuBridge and Brown (1933) amplifier and the photocell B battery are made through a shielded flexible cable, which also carries the galvanometer leads. The amplifier is powered by storage cells (12 volts). A Leeds and Northrup type R galvanometer is used. The entire apparatus is mounted in a dark room, and black felt is used to prevent stray light of the apparatus from reaching the photocell.

Procedure. The image of the lamp filament is sharply focused on the center of the cardboard screen with the dark-field diaphragm removed and the slide in place. It is convenient to place a 12 power convex lens in front of the lamp housing to enlarge the image to about 4 in. The dark-field diaphragm is inserted, and, with the room almost completely dark and the felt curtains in place to eliminate stray light, the image of the ash is focused on the screen with the axial adjustment of the mechanical stage. With the other adjustments of the stage, the desired area is brought to the center of the screen and the photocell box is moved so that the aperture is properly set. Then the shutter is pulled and the galvanometer deflection is observed. The reading obtained when the clear glass of the slide is placed in the optical field is substracted from the first deflection, and the resulting figure is proportional to the quantity of ash on the area taken when this quantity is small. Similar data obtained for other areas enable a comparison of relative amounts of ash without regard to the absolute quantities involved.

F. ANALYTICAL ELECTRON MICROSCOPY

The higher resolving power of the electron microscope has enabled finer morphological differentiations in biological material than were hitherto possible with the best optical microscopes. However important this advantage in other fields, as used today its purely morphological value limits its contributions to cyto- and histochemistry. However, a beginning has been made toward the use of an analytical electron microscope, not merely as a means of obtaining high magnifications, but as a tool for the identification and localization of certain metallic elements in biological preparations. To date only calcium and/or magnesium can be identified and localized. These elements can be detected with a sensitivity of about 1×10^{-12} g. per kilogram wet weight of tissue (muscle). For the

conception of this possibility and the ingenuity to carry through to fruition, credit is due to Gordon H. Scott and his associates, J. H. McMillen and D. M. Parker, who carried out their investigations at Washington University.

Scott and co-workers utilized the well-known fact that when metals or certain of their compounds are heated *in vacuo* they emit electrons whose number depends in part upon both the nature of the metal and the temperature. Hence identification of the metals might be possible on the basis of their differential emission of thermally excited electrons. The localization of these metals in tissue sections would be possible since the electrons emitted could be focused by the magnetic lenses of the electron microscope to yield an image, on a fluorescent screen, of the topographical disposition of the emitting substances.

The first apparatus developed for this purpose was that of McMillen and Scott (1937) but, since a number of changes have been made, only the later apparatus of Scott and Packer (1939a) will be described.

The Scott-Packer Analytical Electron Microscope

A diagram of the instrument is given in Figure 29. Basically it is an electron microscope of relatively low magnifying power fitted at one end with a chamber (C), lined with a material capable of fluorescence by electron streams for the visualization of the electron image, and at the other end with a special cathode (B), carrying a tissue support (A) to hold the paraffin sections that are employed. The microscope tube is made of brass, and is 1 m. long and 63 mm. in diameter. Two magnetic lenses (L_1 and L_2), swung on gimbals, surround the tube and are free to move along it as well as rotate around it to some degree. Each lens is composed of 1550 turns of No. 22 enameled, single-cotton-covered copper wire wound on a copper inner ring 75 mm. in diameter. The coils are enclosed in sheaths of soft iron having a wall thickness of 3 mm. The lenses have an axial width of about 43 mm. It is apparent that the object-image distance is fixed; therefore focusing for any magnification must be accomplished by altering the power of the lenses. This is done by varying the current passing through the lens coils. The power for these coils is supplied from two 30 volt banks of storage cells in parallel.

The tissue holder assembly is made of a 25 mm. glass tube fitted snugly with a brass sleeve soldered to a heavy brass plate. A shoulder on the plate fits into the microscope tube and the joint is rendered vacuum-tight with Apiezon Q sealing compound. Proper centering alignment is maintained by brass cylinders (A and A_1) which fit over the glass tube. The flat top of the nickel cathode cylinder (B) serves as support for the tissue sections and the cylinder itself is held by a wire fixed to its wall. The cathode is heated by a coiled filament (H), which consists of 10 mil tungsten wire supported by a ceramic core not indicated in the diagram. The

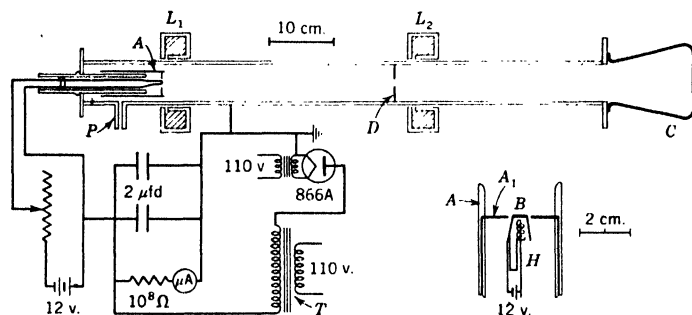



Fig. 29. Diagrammatic sketch of the essential features of the electron microscope. A, tissue holder (cathode) support; A_1, inner shell of same; B, nickel cathode; C, fluorescent screen; D, diaphragm; H, heating filament for cathode; L_1, objective magnetic lens; L_2, ocular magnetic lens; P, pumping port; T, transformer. Other letters and symbols are standard usage in vacuum tube technique. *From Scott and Packer (1939a)*

cathode filament is supplied with current from two 6 volt storage batteries in series which are insulated from ground for 15,000 volts. The insulation is required since the tissue holder assembly and batteries are at a potential of 6000 volts with respect to the grounded microscope tube.

The fluorescent screen (C), which is sealed to the microscope tube with wax, is a commercial oscillograph type. The bare portions of the inner walls are coated with colloidal graphite (Aquadag) to furnish electrical conductivity from the screen to the ground. This prevents the accumulation of charge, which would distort the image on the screen.

The diaphragm (D) has an aperture of 25 mm. over which are placed a few fine wires to serve as fiduciary marks in focusing the lens L_2. The image is formed with lens L_1 (Fig. 30) on the aperture of the diaphragm in order to reduce spherical aberration. The half-wave rectifier and filter system (Fig. 29) supplies the potential difference required for accelerating the electrons. The current passing through the 10^8 ohms resistance placed across the high-voltage leads is measured by the microammeter from which the magnitude of the accelerating voltage can be obtained. The rectifier is supplied by the secondary of a transformer that is insulated from the primary for 15,000 volts. A variable resistance in the primary circuit of this transformer permits the choice of the high D.C. voltage employed.

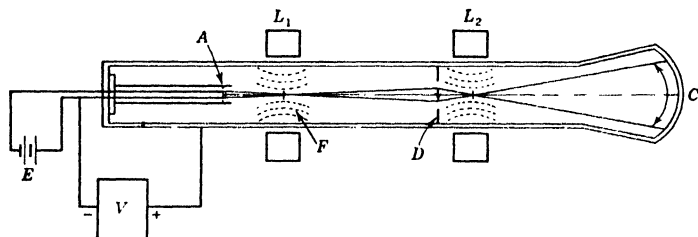



Fig. 30. Diagrammatic representation of the electron path and consequent image formation. *A*, cathode and support; *C*, fluorescent screen; *D*, diaphragm; *E*, batteries for heating filament of cathode; *F*, schema of magnetic field of the lenses L_1 and L_2; *V*, high-voltage source. *From Scott and Packer (1939a)*

Constancy is maintained in the accelerating voltage in order to prevent a distortion, similar to chromatic aberration in optical systems, by the use of a voltage regulator, of the saturated-core transformer type, in the primary circuit of the transformer. Another precaution taken to avoid distortion of the image is the placing of the batteries, high-voltage supply, and all iron objects at quite a distance from the microscope.

In order to compensate, at the magnification used ($< 150\times$), for the deflection of the electron stream by the earth's magnetic field, the first lens (L_1) is tilted.

Water-cooling coils of copper tubing are employed to remove heat from the microscope tube. One coil is wound around the tube on the image side of lens L_1 and another at the junction of the tube and the

object holder. The latter is essential to absorb the 50–60 watts of power given off by the heated filament, which would tend to soften the sealing compound and weaken the vacuum.

The microscope is evacuated through a port (*P*, Fig. 29) by means of two double-stage mercury vapor pumps in parallel employing a Cenco Hyvac oil forepump. A vapor trap cooled with carbon dioxide in butyl alcohol is placed between the microscope and the pumps, and the glass-to-metal connection is sealed with black vacuum wax. Pressures are measured with the ionization gauge of Montgomery and Montgomery (1938) employing a No. 47 radio tube of *Radio Corporation of America.* A portable power pack is used so that it can be employed with several vacuum systems to obviate duplication of expensive meters. A pressure of 10^{-5} mm. mercury or less is sufficient for the electron microscope, and the sensitivity of the pressure gauge is about 7×10^{-6} mm. mercury per microampere ion current.

Manipulation

Preliminary experiments with salts of sodium, potassium, calcium, magnesium, and iron demonstrated that very bright images were formed on the screen by calcium and magnesium, weak ones by sodium and potassium, and none by iron. By maintaining a cathode temperature of 700–800° for an hour the sodium and potassium were volatilized so that the bright image could be safely assigned to calcium and magnesium. For these experiments, ashless gelatin impregnated with the chlorides was hardened in 10% formalin, dehydrated in alcohols and embedded in paraffin. Paraffin sections (10 μ) were placed directly on the prepared cathode.

The nickel cathode is prepared for use by polishing with optical rouge and washing with water and nitric acid. It is then coated with a mixture of 40% barium carbonate and 60% strontium carbonate in a 2% solution of nitrocellulose in amyl acetate. The barium-strontium mixture serves to increase the emission of the calcium and magnesium in the tissue by about 1000%, by activation, and at the same time the mixture emits electrons itself to give a contrast background on the screen. When completely dry, a 10 μ section of tissue embedded in paraffin, prepared by the freezing-drying technique (see page 3), is placed on this surface and smoothed down with a steel needle. The cathode is then inserted into

the microscope tube and the vacuum pumps are started. When the pressure falls to 10^{-5} mm. mercury, or less, the cathode-heating filament is turned on and the temperature is gradually elevated over an hour or more to the operating level. The slow heating is essential to avoid distortions in structure, to minimize or prevent curling on the cathode, and to approximate microincineration conditions in order to permit a comparison. The practice is followed of heating the cathode higher than operating temperature for a short time to volatilize the sodium and potassium and to initiate active emission of electrons. When this activation period is over, and the operating temperature obtained, the high-voltage and lens currents are turned on. The electron image formed on the screen is compared with stained or incinerated control sections. When the tissue curls away from the cathode and is improperly oxidized, dark areas appear in the image and carbonization is evident by optical examination. The magnification is altered by changing the position of the magnetic lenses on the tube and then bringing to focus by adjustment of the lens current. Electron-accelerating voltages of 5000–6000 were employed. Various portions of the section are focused on the center of the screen by virtue of the mobility of the lenses.

Photographs of the image are made with a high speed camera ($f = 2.9$) at a camera magnification of 0.73 on Eastman Kodak Superspeed Ortho Portrait or Panchro-Press film. Exposures of 1–5 sec. are usually employed. The films are developed with Eastman Kodak D-72 developer. Some photographs obtained in this fashion are shown in Figures 31 and 32.

G. RADIOAUTOGRAPHY

The novel and thus far very limited technique of radioautography has been employed increasingly for the localization of radioactive elements in tissue sections. The use of these isotopes as tracers in biochemical investigations, particularly as the result of the pioneering work of Hevesy, is a well-established device; however histological distribution cannot be determined quantitatively, as yet, on the basis of radioactivity, by any very satisfactory means, since the order of the intensity of the radiation produced in the quantities of tissue commonly employed for histological examinations is far too small to permit suitable measurements with the

Geiger-Müller counter or the electroscope. Radioautography, based on the ability of emanations from radioactive elements to affect the photographic plate, is an attempt toward the solution of this difficult problem. Tissue sections containing radioactive elements leave their "autographs" on photographic plates when placed in contact with

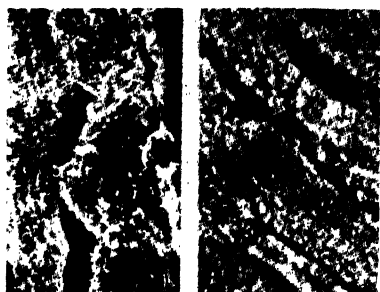



Fig. 31. Emission electron micrograph (×300) showing calcium and magnesium distribution in rectus abdominus muscle of cat. Note strong cross-bandings in muscle fibers. *From Scott (1943)*

Fig. 32. Emission electron micrograph of cat gastric mucosa (fundus) showing calcium and magnesium distribution (left), compared with a microincinerated section from the same animal (right). Magnification about ×75. *From Scott (1943)*

them for a sufficient period. When developed, these "autographs" indicate to some degree the relative distribution of the substances responsible for the radioactivity.

Historically, the first use of radioautography was made by Lacassagne and Lattès (1924) for the demonstration of polonium in tissue. Since that time the usefulness of this technique, as well as all others employing radioactive tracers, has been greatly expanded by the recent revolutionary developments which have made possible the preparation of radioactive isotopes of elements that occur in living systems. Limiting factors in regard to the suitability of a radioactive isotope for studies by radioautography are the nature and intensity of its radiation and its half-life period. The duration of the photographic exposure will depend on these factors as well as on the concentration of the isotope in the tissue. The half-life periods of the principal artificial radioactive elements that might be used in tracer studies are given in Table V.

Perhaps, the greatest deficiency of the technique of radioautogra-

TABLE V. Principal Artificial Radioactive Isotopes Used as Trace Elements as Compiled by Pool and Kurbatov (1943)

Radioactive element	Half-life	Atomic weight	Intensity of activity	Type of radiation
O	2.1 min.	15	Strong	$+\beta$
N	9.93	13	Strong	$+\beta\gamma$
Mg	10.0	27	Strong	$-\beta\gamma$
Co	11.0	60	Strong	$-\beta\gamma$
C	21.0	11	Strong	$+\beta$
Ag	24.5	106	Strong	$+\beta$
I	25.0	128	Strong	$-\beta\gamma$
Cl	37.5	38	Strong	$-\beta\gamma$
In	54	116	Strong	$-\beta\gamma$
Zn	57	69	Strong	$-\beta$
Ba	1.42 hr.	139	Strong	$-\beta\gamma$
F	1.8	18	Strong	$+\beta$
Se	1.81	75	Weak	$+\beta$
Y	2.0	88	Strong	$+\beta$
Cr	2.27	55	Weak	$-\beta$
Mn	2.59	56	Strong	$-\beta\gamma$
Si	2.60	31	Strong	$-\beta$
Ni	2.6	63	Strong	$-\beta\gamma$
Ti	3.1	45	Strong	$+\beta$
Sc	4.0	43	Strong	$+\beta\gamma$
Br	4.45	80	Weak	γ
Ab	7.5	211	Weak	$\alpha\gamma$
K	12.4	42	Strong	$-\beta\gamma$
I	12.6	130	Strong	$-\beta\gamma$
Cu	12.8	64	Strong	$-\beta$, $+\beta$
Au	13.0	196	Weak	$-\beta$
Pd	13.0	109	Weak	$-\beta$
Zn	13.8	69	Weak	γ
Na	14.8	24	Strong	$-\beta\gamma$
Pt	18	197	Weak	$-\beta$
Co	18.2	55	Strong	$+\beta\gamma$
W	1.01 day	187	Weak	$-\beta\gamma$
Sn	1.05	121	Weak	$-\beta$
As	1.11	76	Strong	$-\beta$, $+\beta\gamma$
La	1.70	140	Strong	$-\beta\gamma$
Ni	1.5	57	Weak	$+\beta$
Br	1.66	82	Strong	$-\beta\gamma$

TABLE V (*Concluded*)

Radioactive element	Half-life	Atomic weight	Intensity of activity	Type of radiation
Cd	2.3 days	115	Strong	$-\beta\gamma$
Au	2.7	198	Weak	$-\beta\gamma$
Mo	2.8	99	Weak	$-\beta$
Ag	7.5	111	Strong	$-\beta$
I	7.8	131	Strong	$-\beta\gamma$
Ca	8	41	Weak	γ
Ag	8.2	106	Strong	γ
Sn	10.0	123	Weak	$-\beta$
P	14.5	32	Strong	$-\beta$
V	16	48	Weak	$+\beta\gamma$
As	16	74	Strong	$-\beta, +\beta\gamma$
Rb	19.5	86	Strong	$-\beta$
Cr	26.5	51	Weak	γ
Be	43	7	Weak	γ
Fe	47	59	Weak	$-\beta\gamma$
Sr	55	89	Strong	$-\beta$
Sb	60	124	Strong	$-\beta\gamma$
Zr	63	93	Weak	$-\beta$
Ti	72	51	Weak	$-\beta\gamma$
W	74.5	185	Weak	$-\beta$
Sc	85	46	Strong	$-\beta\gamma$
S	88	35	Weak	$-\beta$
Ta	97	182	Weak	$-\beta\gamma$
Y	105	86	Strong	γ
Ca	180	45	Weak	$-\beta\gamma$
Zn	250	65	Weak	$+\beta\gamma$
Mn	310	54	Weak	γ
Cs	1.7 yr.	134	Weak	$-\beta\gamma$
V	1.71	47	Weak	γ
Na	3.0	22	Weak	$+\beta\gamma$
Co	5.3	60	Weak	$-\beta\gamma$
H	31	3	Weak	$-\beta$
Ra	1590	226	Strong	α
C	10000	14	Weak	$-\beta$
U	7.1×10^8	235	Weak	α
U	4.5×10^9	238	Weak	α
Rb	1×10^{11}	87	Weak	$-\beta$

phy lies in its inability to reveal distribution in the finer structures, and to this lack of resolving power must be added the further drawback that, quantitatively, only a rough approximation is possible. However, there is the considerable advantage, inherent in the use of radioactive elements regardless of whether a histological or gross tissue study is involved, that very small quantities of an element introduced into a biological system can be followed without reference to, or interference from, the large stores normally present. The amount of a radioactive element that can be detected is fortunately, very minute. According to Hamilton (1941) a total of 2×10^6 β particles, with an average energy of at least 150 Kev., are required to strike each cm.^{2} of photosensitive surface to yield a satisfactory image.

Reviews dealing with radioautography have been presented by Hamilton (1941–1942) and Simpson (1943), and important physical data have been furnished in a review by Kurbatov and Pool (1943).

Preparation of Radioautographs*

Both fresh-frozen and paraffin sections of tissue have been used to obtain radioautographs. In general the paraffin sections give the best results since they can be cut thinner and are less subject to distortion. It is essential that the sections be of uniform thickness and free of wrinkles. There would be a particular advantage in the use of freezing dehydration (page 3) for the preparation of the paraffin-infiltrated tissue. The diffusion of the radioactive substances during fixation and dehydration in solutions would be eliminated and a more authentic "autograph" could be obtained.

As examples of procedures which have been used the following may be cited: Hamilton, Soley, and Eichorn (1940), in a study of radioactive iodine in thyroid tissue, removed the paraffin from 3–5 μ sections with xylol, dipped the slide containing the sections in dilute celloidin, allowed it to dry, and obtained a celloidin film over the sections about 1 μ thick. The sensitive surface of the photographic film was placed in contact with the celloidin surface. Harrison, Thomas, and Hill (1944) in an investigation of the distribution of

* See Bibliography Appendix, Refs. 20, 21, 22, 28, and 30.

radioactive sulfur in the wheat kernel, employed paraffin sections 25–50 μ thick which were covered directly by a layer of aluminum foil 0.8 μ thick. The sensitive photographic surface was placed in contact with the foil.

The greater the distance between the tissue and the photosensitive surface, the poorer the resolution in the radioautograph due to scattering of the radiation. It is preferable that this distance be kept under 1 mm. Ultraspeed x-ray film has been extensively used, but it has the disadvantage of producing grainy enlargements. Harrison, Thomas, and Hill (1944) recommend a fine grained panatomic film when it is possible to have longer exposures. The photographic film over the sections on a glass slide is covered with another slide and the whole bound firmly together with cellulose tape. All of these operations are carried out in a dark room, of course. After wrapping the slide in light-tight black paper, it may be placed in a cold place for the duration of the exposure. It is advisable to keep the sections cold to inhibit any tendency toward diffusion of the radioactive element. After exposure, the sections are stained in the usual manner to bring out their morphology, and compared to the developed "autographs" with the aid of a dissecting microscope.

More recently, Bélanger and Leblond (1946) extended the usefulness of radioautography by the ingenious expedient of spreading a photographic emulsion directly on the sections. This not only permits a more intimate contact between the tissue and the photographic surface, but it obviates the matching of the "autograph" to the corresponding histological detail, which is particularly difficult at higher magnifications. The possibilities of this technique merit a more detailed description of the procedure.

Bélanger and Leblond Technique

Preparation of Photographic Emulsion. Soak lantern slide plates (medium contrast, Eastman) in distilled water at room temperature. When the gelatin swells, remove from the water. With a glass knife scrape off the gelatin, and melt it in a beaker placed in a 35–40° bath. Carry out this procedure and all others in which the emulsion is used in a dark room. A Wratten "Safelight—No. 1" (Eastman) may be used at a distance of about 3 ft.

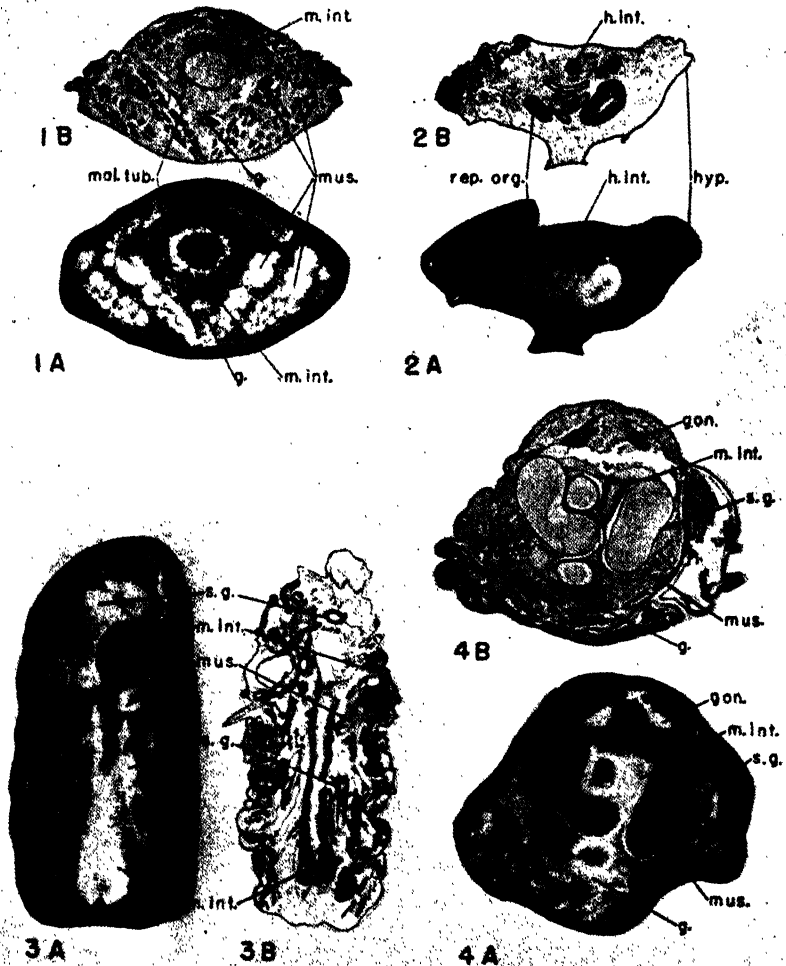



Fig. 33. Radioautographs (A) and corresponding stained sections (B) (×8). White areas in radioautographs are exposed parts of film. (1) Thorax of adult mealworm, transverse section, paraffin; (2) abdomen of adult mealworm, transverse section, paraffin; (3) abdomen of wax moth larva, longitudinal section. frozen; (4) abdomen of wax moth larva, transverse section, frozen; (g) ganglion; (gon.) gonad; (hy.) hypodermis; (mal. tub.) malpighian tubule; (m. int.) midintestine; (mus.) muscle; (h. int.) hind intestine; (rep. org.) reproductive organs; (s. g.) silk gland. *From Lindsay and Craig (1942)*

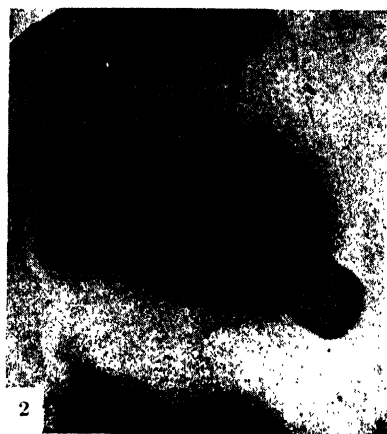
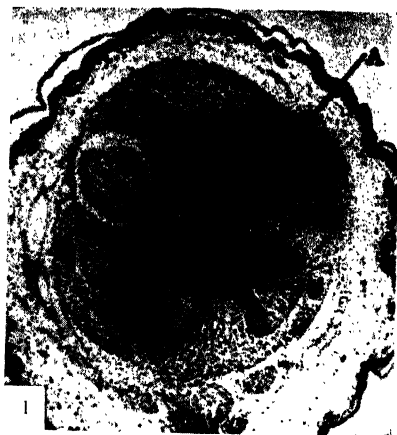



Fig. 34. Radioautographs of adult rat tissues. (*1*) Cross section through the lower limb (×50). The radiophosphorus is in the diaphysis of the tibia and the fibula. Arrow A points to heavy periosteal layer in the tibia. (*2*) Paramedian longitudinal section of thoracic vertebra (×50). Arrow B indicates phosphorus deposit in the ossifying neural arch. (*3*) Cross section of the lower jaw (×17). The deposition of radiophosphorus clearly outlines the mandible. In the right portion of the bone an incisor tooth is developing and is also impregnated with the radioactive element. (*4*) Section of the thyroid from an adult rat (×70), treated with radioiodine. The tracheal cartilage is visible at the bottom of the figure. The thyroid follicles show a reaction due to radioiodine. *From Bélanger and Leblond (1946)*

PROCEDURE

1. Prepare 10 μ paraffin sections and attach to slides with egg albumin.
2. Dry, deparaffinize with xylol, and carry through absolute alcohol to 1% celloidin in alcohol-ether.
3. Drain off excess celloidin by standing the slides in an empty Coplin jar.
4. Place in 70% alcohol for about 1 min. to harden the celloidin, and dry at room temperature.
5. In the dark room, pipette 5 drops of the melted photographic emulsion on to each slide and spread evenly with a camel's hair brush. Carry out this operation a little below 40° on a hot plate to prevent premature gelling of the emulsion. (The temperature should be held below 40° with this emulsion to reduce the fogging.)
6. Allow to cool and dry, and place the slides horizontally in a dustproof, light-tight box for the duration of the exposure.
7. After the exposure develop for 3–4 min. in Kodak developer D72 at 18–20°. Wash rapidly in water and fix for about 10 min. in 5% thiosulfate at the same temperature. Wash finally in cold running water for about 30 min. The black silver deposit indicates the site of the radioactive element.
8. Counterstain in Coplin jars cooled in running water. (Methylene blue and Harris hematoxylin may be used for radiophosphorus "autographs" and Harris hematoxylin for those of radioiodine.) With methylene blue place slides in a 1% alkaline soln. for about 30 min. and rinse in running water until the stain is removed from the gelatin coating. With Harris hematoxylin, stain lightly to avoid the need of differentiating. (Artifacts caused by gelatin swelling and disengagement of the sections disappear when the slides are thoroughly dried after each operation.)
9. Pass through several changes of 95% alcohol, absolute alcohol, and xylol, and mount in Canada balsam. (Clearing in oil of origanum before mounting will also give good results).

Discussion

Points on various procedures required for particular studies may be obtained by referring to some of the applications already made. To date, most of the investigations employing radioautography

deal with the isotope P^{32}. Thus its distribution was studied in bones by Dols *et al.* (1938) and Bélanger and Leblond (1946), in tomato fruits by Arnon *et al.* (1940), in squash plants by Colwell (1942), and in insects by Lindsay and Craig (1942). "Autographs" have been obtained in bone studies with radioactive calcium and strontium by Pecher (1941) and Treadwell *et al.* (1942). The distribution of radioactive lead in the animal body was investigated by Behrens and Baumann (1933a,b), and thyroid studies were carried out with radioactive iodine by Hamilton *et al.* (1940), Gorbman and Evans (1941), and Bélanger and Leblond (1946). Harrison, Thomas, and Hill (1944) employed radioactive sulfur for a radioautograph survey of the distribution of this element in wheat. Many new applications are constantly appearing.

CHEMICAL TECHNIQUES

"By calling attention to the cell I desired to provoke investigators to inquire into the processes within the cell, to define that which happens within these smallest elementary organisms. And it was self-evident that an exact definition could be nothing else than to find the chemical and physical foundations upon which vital phenomena and the activity of the cell are based."

VIRCHOW

as quoted by Paul Klemperer in *Some Recent Biologic Investigations and Their Significance for Pathology,* J. Mt. Sinai Hosp. N. Y. 14: 442 (1947/48).

INTRODUCTION

The chemical techniques to be described are all of the quantitative variety and they differ from their macro counterparts primarily as regards the volumes employed and the mode of handling them. In general, the same reactions and concentrations of reagents are used in both. The degree to which the localization of the chemical constituents in tissues and cells is limited, in these techniques, largely depends upon the degree to which the anatomical parts can be isolated mechanically in preparation for their separate analysis. The precedures most commoly used are: (*a*) the preparation of serial microtome sections of tissue and analysis on each of selected sections, (*b*) isolation of cells or cellular particulates by centrifugation for their separate analyses, or (*c*) use of micro dissection to obtain the part to be analyzed. It is considerably more of a problem, as a rule, to obtain a satisfactory sample for analysis than to perform the analysis itself. While the ultimate goal of being able to apply quantitative procedures *in situ* to biological material is still essentially beyond the present horizon, the use of these chemical techniques can lead to the acquisition of knowledge which can now be obtained by no other means.

It should be pointed out that in the interests of simplicity and accuracy certain well-established procedures of macroquantitative analysis are best avoided in work on the level considered here. The procedures to be given are those of the original authors, but the laboratory worker should introduce his own simplifications of the following type at every opportunity: (*1*) Avoid quantitative transfers—rather remove an aliquot. (*2*) Avoid dilution to a given volume in a vessel; this necessitates the calibration and marking of the vessel—rather dilute by adding a known volume of liquid with a pipette. (*3*) Employ pipettes calibrated to deliver rather than to contain—this obviates the necessity of rinsing the pipette. (*4*) Avoid filtration when it is possible to separate a precipitate by centrifugation.

I. GENERAL APPARATUS AND MANIPULATION

A. VESSELS, STOPPERS, HOLDERS, ETC.

Vessels. Most of the reactions employed in the various chemical techniques are carried out in simple glass vessels. The tube shown in Figure 35 is especially useful; it is nothing more than a small test tube having a total capacity of 0.25 ml. (available from *A. H. Thomas Co.* and E. Petersen, Carlsberg Laboratory, Copenhagen Denmark). Norberg (1937) employed the tubes (Fig. 36) for use in centrifugation and the apparatus shown in Figure 37 for removal of supernatant fluid from centrifuged precipitates. By applying suction at *A* the fluid is drawn into the reservoir; the low-power microscope is used to enable careful control of the operation. As indicated in Figure 37, the tube may be surrounded by a larger vessel filled with a clear liquid, such as alcohol, to permit better observation, particularly when the vessel on the left (Fig. 36) is used, since the bottom part of the tube has a dark zone due to its form.

The tubes may be cleaned conveniently by immersing in the cleaning liquid, heating to drive the air out of the tubes, cooling to let them fill up with the liquid, and shaking out the liquid from each one. Usually the process is repeated two or three times. To place films of liquid across the upper portion of a reaction tube, as in iodometric titrations, Holter and Doyle (1938) employed the vessel shown in Figure 38, which has a total volume of 0.20 ml. These vessels must be given an inner hydrophobic coating to prevent the liquid from spreading on the glass surface. The method of doing this is described on page 169.

Levy (1936) used the 2.5 ml. tube illustrated in Figure 39 for the Kjeldahl digestions and Linderstrøm-Lang, Weil, and Holter (1935) employed the two-piece unit shown in Figure 40 for ammonia dis-

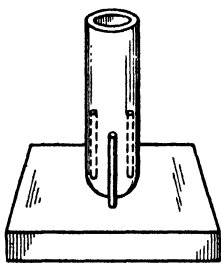



Fig. 35. Reaction vessel in holder. *From Linderstrøm-Lang and Holter (1933a)*

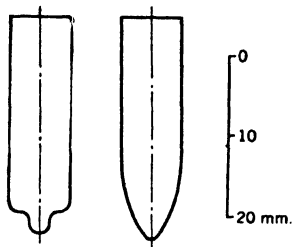



Fig. 36. Centrifuge reaction vessels, *From Norberg (1937)*

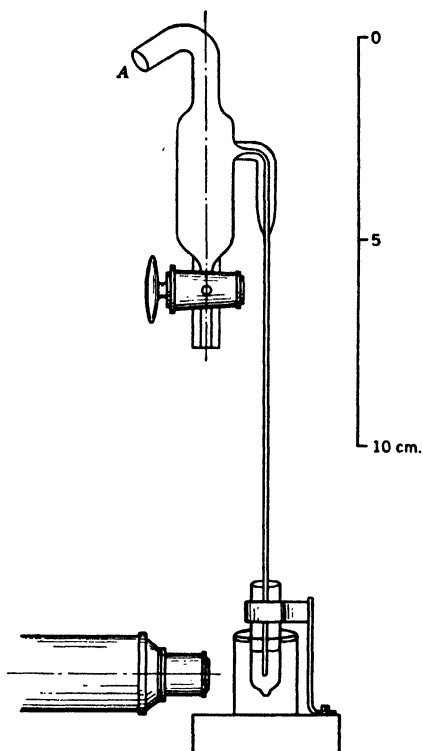



Fig. 37. Arrangement for removing supernatant fluid over a precipitate. *From Norberg (1937)*

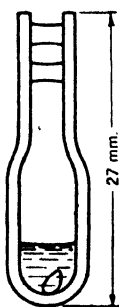
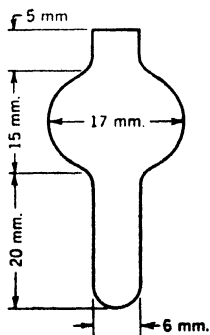
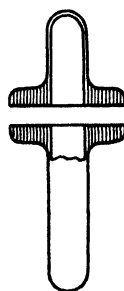



Fig. 38. **Fig. 39.** **Fig. 40.**

Fig. 38. Reaction vessel for iodometric titration. In neck, lower film is sulfuric acid, upper film starch solution. ***From Holter and Doyle (1938)***

tillations, *e.g.*, in arginase measurements. The ammonia was distilled from the vessel into the cap, which was coated internally with paraffin and charged with standard acid. Ramsay grease (thick) was used to seal the parts together. Later it was found preferable to abandon the use of this form of vessel for ammonia distillation (Brüel *et al.*, 1946) (see page 283).

Glass diffusion cells for the distillation of ammonia were described first by Conway and Byrne (1933), and later by others (Figs. 41–43). Ammonia diffuses from the outer well into standard acid con-

Fig. 42. Gibbs and Kirk (1934) diffusion cell (cross section, one half actual size).

Fig. 41. Conway and Byrne (1933) diffusion cell. Above, top view; below, vertical, section on line *AB*.

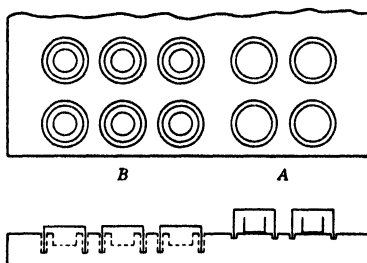



Fig. 43. Kinsey and Robinson (1946) diffusion cells: upper, top view; lower, side view.

tained in the center well in the types shown in Figures 41 and 42 (available from *Microchemical Specialties Co.*). The Kinsey and Robison (1946) apparatus (Fig. 43) consists of a Lucite plate 0.5 in. thick with rings 1 mm. deep having inner and outer diameters of 14 and 18 mm., respectively, reamed out of the plastic for one form of cell (*A*); for the other form (*B*), rings of the same diameter but 8 mm. deep are reamed out and a center hole 4 mm. deep and 8 mm. in diameter is drilled. In the *A* form cell, two glass vials are used alone. Ammonia diffuses into the receiving solution placed in the

bottom of the outer or larger vial. When the vial is inverted and set on the plate this solution forms a hanging drop over the liquid which is liberating ammonia.

A small open porcelain dish (*Microchemical Specialties Co.*) (Fig. 44 below) was used by Kirk and associates as a titration vessel.

Fig. 44. Titration dish, actual size. *From Kirk and Bentley (1936)*

Coating Vessels with a Hydrophobic Layer. At times it is desirable to coat reaction vessels with a hydrophobic layer to prevent aqueous liquids from spreading on the glass surface, as in iodometric titrations where liquid films are placed across the lumen of the neck of the titration tube. Linderstrøm-Lang and Holter (1933a) used paraffin and Holter and Doyle (1938) employed ceresine. The procedure followed by the latter was to boil about 50 vessels for 5–10 min. in 75 ml. of water to which 0.1 g. ceresine was added. After the water had cooled, the vessels were emptied and dried for at least 3 hr. at 100–110°.

The procedure finally employed at the Carlsberg Laboratory for paraffin coating was described by Brüel *et al.* (1946). The clean, dry glass tubes are immersed in melted paraffin at 150–200° (the synthetic paraffin used had a melting point of 82°), picked out one at a time with forceps, quickly emptied and rotated in a clean towel between the fingers until the paraffin solidifies. A heavy layer of paraffin on the bottom of the tube and a thinner one on the upper part is desirable. The outside of each of the tubes is wiped free of paraffin and they are stored protected from dust and fumes. After the vessels have been used, they are cleaned by rinsing first with water, then with acetone, hot toluene, acetone, and water in the order given.

Stoppers. For most work it is sufficient to stopper reaction tubes with a short piece of rubber tubing one end of which is plugged with a glass bead or short piece of glass rod. A stopper consisting of a cap with a small hole (Fig. 45) is useful in some cases as in the addition of alkali in the method of Linderstrøm-Lang and Holter (1933b) for ammonia. In this same method a stopper was used

having a drawn-out piece of glass tubing to plug one end (Fig. 46) so that the larger air space would prevent the displacement of the liquid film, which was across the tube, when the stopper was fitted on. To protect solutions from atmospheric carbon dioxide, Linderstrøm-Lang, Weil, and Holter (1935) employed stoppers containing soda lime tubes (Fig. 47).

Tube Holders. Perhaps the simplest holder for a small reaction tube is a short length of thick-walled rubber tubing into which the bottom of the tube may be placed, as in Figure 50. It is more convenient to use a small wooden or metal block with three flexible metal prongs to hold the tube. For titration, where the color of the solution is to be matched with a color standard, a single block with prongs to hold two tubes is used (see Fig. 64, page 180; *A. H. Thomas Co.* and E. Petersen, Carlsberg Laboratory).

Reductor. Kirk and Bentley (1936) devised a small glass volumetric flask of either 0.1 or 0.2 ml. capacity for use as a reductor in their method for the estimation of iron (Fig. 48). The reductor is made from heavy-walled 2 mm. bore capillary tubing. In the iron method (page 277) cadmium amalgam is employed to reduce the iron.

Tube and Pestle. For the grinding of bits of tissue, Glick (1937) used a small pestle with a 250 μl. tube having the inner bottom surface ground as shown in Figure 49.

B. MICROLITER PIPETTES

Pipettes of various designs have been employed for measuring microliter volumes. The chief among these will be described.

Fixed Pipettes. One of the pipettes developed by Linderstrøm-Lang and Holter (1931) is shown in Figure 50 (*A. H. Thomas Co.* and E. Petersen, Carlsberg Laboratory). It consists of a capillary tube drawn out to a tip which is slightly bent so that contact can be made with the wall of a vessel. The pipette is calibrated by first weighing it, and then filling it with water to a little more than the required volume. The pipette is again placed on the pan of the balance and water is removed by touching a piece of filter paper to the tip. When the desired weight of water remains in the pipette, it is removed from the balance and a mark is placed at the meniscus, either by etching with hydrofluoric acid or using a piece of gummed

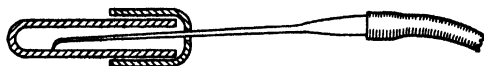



Fig. 45. Reaction tube cap with hole. *From Linderstrøm-Lang and Holter (1933b)*

Fig. 46. Stopper for reaction tube with cap of drawn-out glass tubing. *From Linderstrøm-Lang and Holter (1933b)*

1 cm.
Ground surface
Ground inner surface

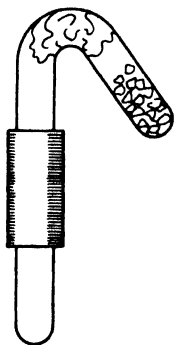
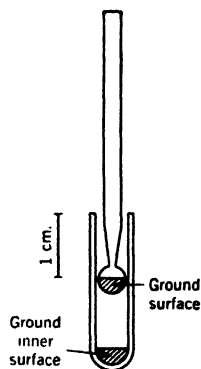



Fig. 47. Fig. 48. Fig. 49.

Fig. 47. Soda lime tube for stoppering reaction vessel. *From Linderstrøm-Lang, Weil, and Holter (1935)*

Fig. 48. Reductor, actual size. *From Kirk and Bentley (1936)*

Fig. 49. Tube and pestle for grinding tissue. *From Glick (1937)*

paper. In the assembly shown in Figure 50, the pipette is filled by applying gentle suction through the tube *S* with *H* closed and *K* open. When the liquid is a little above the mark, *K* is closed and the slowly falling meniscus is observed through the low-power microscope (*M*). The moment the meniscus reaches the mark, the vessel of liquid is quickly lowered away from the tip and the capillary forces will prevent the liquid from running out of the pipette. The vessel into which the liquid is to be delivered is brought up so that the pipette tip touches the vessel wall near the bottom and *H* is opened. *P* leads to a source of compressed air, and the pressure regulator (*T*) enables the liquid to be forced out of the pipette under constant pressure. Usually a 20 cm. column of water gives the required pressure; the emptying time should not be less than 5 sec. *H* is not to be closed until the delivered liquid has been lowered away from the pipette. With pipettes having a capacity of 7 μl. the error of pipetting was found to be less than 0.3%.

Hand Pipettes. A hand pipette (*A. H. Thomas Co.* and E. Petersen, Carlsberg Laboratory), Figure 51, having an accuracy of about 1% was also used by the Carlsberg group. The instrument is filled or emptied by sucking or blowing through the attached rubber tubing. The tip of the pipette is fine enough to prevent liquid from running out unless a slight pressure is applied through the rubber tubing. Hand pipettes, in which the suction or pressure is applied by a glass syringe, have been used by Kirk's group, Kirk and Craig (1932), Sisco, Cunningham, and Kirk (1941) (Figure 52) (*Microchemical Specialties Co.*). A rubber gasket fixed to the end of the syringe barrel receives the large end of the pipette, or the metal syringe fitting of a hypodermic needle is cemented to the pipette in order to permit easy attachment to, and separation from, the syringe.

Constriction Pipettes. The preceding types of pipette have been displaced very largely by the constriction pipette (Levy, 1936; Linderstrøm-Lang and Holter, 1940) shown in Figure 53 (*A. H. Thomas Co.* and E. Petersen, Carlsberg Laboratory). In this pipette the calibration mark is replaced by a constriction in the lumen of the capillary. Liquid is first sucked up over the constriction and a slight pressure is then applied which causes the liquid to fall down to the constriction but not past it. To deliver the charge, a momentary greater pressure is applied to force the meniscus through the constriction and then gentle pressure can be employed to empty the

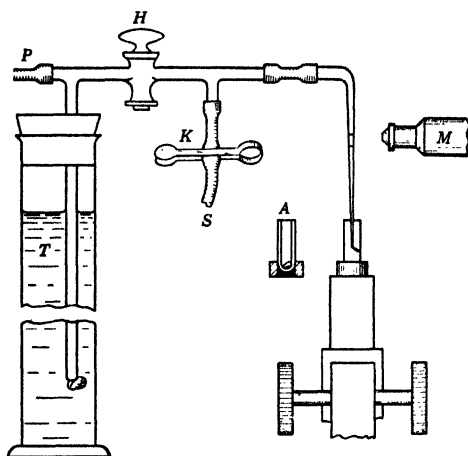



Fig. 50. Fixed pipette.
From Linderstrøm-Lang and Holter (1931)

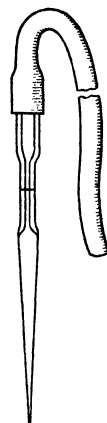



Fig. 51. Hand pipette.
From Linderstrøm-Lang and Holter (1933a)

Fig. 52. Capillary pipette and syringe control. *From Sisco, Cunningham, and Kirk (1941)*

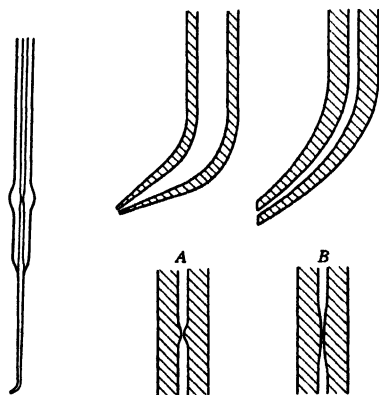
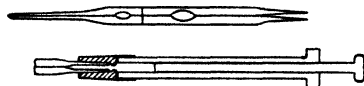



Fig. 53. Constriction pipette:
(A) proper form; (B) faulty form.
From Linderstrøm-Lang and Holter (1940)

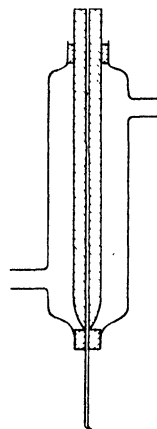



Fig. 54. Constriction pipette with water jacket.
From Holter and Doyle (1938)

pipette. If employed in the assembly shown in Figure 50, a quick squeezing of the rubber tubing over *K* will be sufficient to initiate the emptying process. Greater accuracy is obtained by adjusting the dimensions so that the pipette delivers automatically without applying excess pressure when the tip touches the vessel or the liquid. The tip and the constriction should be constructed as in *A* (Fig. 53), and not as in *B*. Holter and Doyle (1938) employed a constriction pipette surrounded by a water jacket to control the temperature of the liquid being pipetted (Fig. 54).

In the procedure for the determination of total nitrogen (pages 234 and 283) the pipettes used must meet certain dimensional requirements as defined by Brüel *et al.* (1946). Thus, the pipette used to transfer the digested sample must have a tip, the opening of which is not so narrow as to become blocked by small crystals or other particles; but neither must it be so wide as to make it difficult to empty the pipette without blowing air through the tip, which might cause spattering of the liquid delivered. Furthermore, the pipette stem must be thin enough for use in a narrow tube without causing the liquid to be drawn up between the tube and pipette, and yet it must be thick enough to have mechanical strength. A suitable pipette is illustrated in Figure 55, and the allowable variation in the dimensions is shown in Figure 56, which gives the dimensions of a rather thin and a rather thick pipette, either of which may be used.

The dimensions of a suitable constriction pipette for pipetting the acid used to absorb ammonia are given in Figure 57.

Placing a water seal of known volume across the lumen of a reaction tube is best performed with the type of constriction pipette shown in Figure 58. Water is drawn up to the point *X*; the entire amount is blown out to form the seal, and then the excess water is sucked back into the pipette to *Y*. The amount left in the seal is then the volume between *X* and *Y* in the pipette. Acid-selenium mixture is pipetted with the horizontal pipette illustrated in Figure 59. Each division corresponds to 1 μl. These pipettes are available from E. Petersen, Carlsberg Laboratory.

Automatic Pipettes. An automatic pipette was designed by Linderstrøm-Lang and Holter (1931) and it was used by them for the accurate delivery of 20–40 μl. alcoholic acid to stop enzyme action (Fig. 60) (*A. H. Thomas Co.* and E. Petersen, Carlsberg Laboratory). The pipette consists of a narrow glass tube drawn out to a

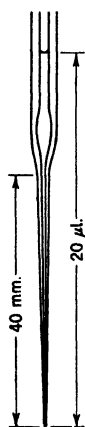
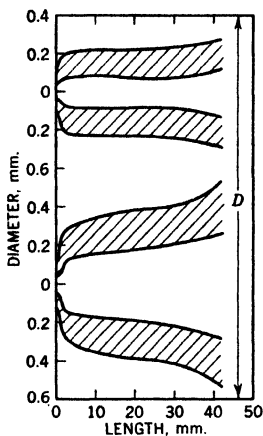
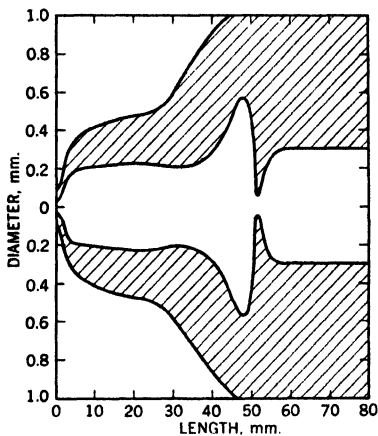



Fig. 55. Pipette for transfer of digested sample in nitrogen determination. *From Brüel et al. (1946)*

Fig. 56. Allowable variation in dimensions of pipette in Fig. 55. *From Brüel et al. (1946)*

Fig. 57. Dimensions of constriction pipette for pipetting acid used to absorb ammonia in nitrogen determination. *From Brüel et al. (1946)*

Fig. 59. Horizontal mouth-operated pipette with vertical delivery tip. *From Brüel et al. (1946)*

Fig. 58. Constriction pipette for placing liquid seal across neck of reaction tubes.

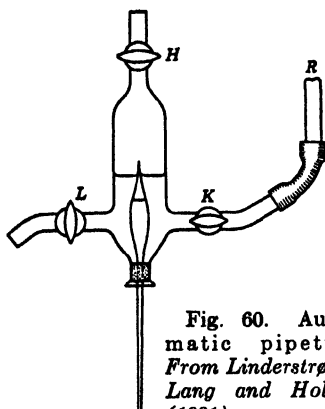



Fig. 60. Automatic pipette. *From Linderstrøm-Lang and Holter*

capillary at both ends so that, with one atmosphere pressure, the fineness of the tips will prevent liquid from running out. *R* is a siphon arm connecting to a reservoir of the liquid to be pipetted which is placed about 50 cm. above the instrument. The pipette is filled as follows: Close *L* and open *K* and *H* to fill the outer chamber. When the level is a few mm. over the upper tip of the pipette, close *H*; the pressure from the reservoir will fill the pipette. Then close *K* and open *H* and *L* to bring the level of the liquid in the outer chamber below the upper tip. Deliver the pipette charge by closing *H* and *L* and opening *K*, which compresses the air in the chamber and forces the liquid out. A pipette of this type having a capacity of 30 μl. was found to have an error of measurement of less than 0.1%.

Accurate syringe pipettes have been employed which use a screw (Krogh and Keys, 1931; Krogh, 1935) or a micrometer spindle (Trevan, 1925) to move the plunger of a small hypodermic syringe. The micrometer syringe pipettes are essentially the same as the micrometer burettes of Dean and Fetcher (1942) and Hadfield (1942) (page 255). The Krogh-Keys instrument is manufactured by *Macalister Bicknell Co.* It has a delivery precision of 0.1 μl.

Devices for Drawing Finer Pipettes. Finer pipettes which are used under a microscope may be drawn by hand, but mechanical devices for making them are considerably more efficient. DuBois (1931) described an automatic device for drawing very fine micropipettes and microneedles which has been made available commercially (Leitz). A capillary tube is clamped in two arms of the device, and between the arms the tube passes through a small electric heater. When the glass softens in the heater the spring tension on the arms pulls them back, thus drawing out the tube into a pair of pipettes. Rachele's device, described by Benedetti-Pichler and Rachele (1940), operates in a similar manner except that gravity is used to pull out and lower the arms when the glass is softened by the electric heater.

C. FILTERS

Sintered-glass filters for small volumes of liquid were used by Kirk and co-workers for the quantitative collection of precipitates for various determinations. The filter described by Cunningham, Kirk, and Brooks (1941b) is made of capillary tubing (2 mm. in-

ternal diameter, 6 mm. external diameter) the end of which is tapered and contains a fused-in 4 mm. section of sintered glass at the tip. Kirk (1935) discussed the preparation of sintered-glass filters for those who cannot obtain them commercially. A layer of fine asbestos 1 mm. thick is sucked onto the tip of the filter to form a pad; after pressing this pad down with the fingernail, a layer of asbestos is deposited on the pad to form a cone about 2 mm. deep The filter is used as shown in Figure 61. Only the tip of the cone is allowed to be in contact with the liquid in order that the precipitate may be collected entirely on this part of the asbestos. The precipitate can be transferred quantitatively by disengaging the pad at its base. These filters are available from *Microchemical Specialties Co.*

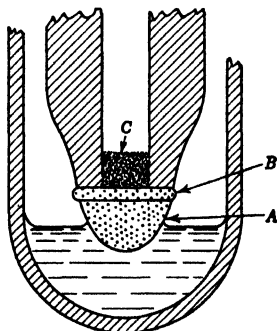



Fig. 61. Filtration detail. *A* represents an asbestos filtering cone; *B*, an asbestos base pad; and *C*, a sintered-glass plug. *From Cunningham, Kirk, and Brooks (1941b)*

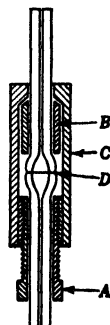



Fig. 62. Ultrafilter for small volumes of liquids. *From Johnson and Kirk (1940)*

Bott (1943) employed a capillary tube filter with paper pulp (Fig. 76) in the determination of sodium. A description of the preparation of filter and its use is given on pages 204–207.

Ultrafilters. Various devices have been employed for the ultrafiltration of small volumes of liquids, and only that of Johnson and Kirk (1940), designed for about 0.1 ml., will be described, since it is one of the simpler and more efficient types. The ultrafilter shown in Figure 62 consists of two brass tubes, *A* and *B*, drilled to fit snugly the glass capillary tubes having a bore of 2 mm. or less and an outside diameter of 7–8 mm. Krönig cement (1 part white wax and 4

parts rosin melted together) is used to bind the glass to the metal. A brass collar, *C*, engages *B* and is threaded to *A*. For visibility, the center part of the collar is cut away on two sides. The ends of the capillary tubes are flared and ground flat, and a piece of collodion membrane is held tightly between the ground surfaces at *D*. The ultrafilter is used with positive pressure.

D. STIRRING DEVICES

Many investigators have used a stream of carbon dioxide-free air bubbles ejected from a fine glass tip to obtain stirring in manipulations of a submacro order. However, when very small volumes of liquid are to be stirred, recourse must be had to other means. When dealing with a drop of liquid in a capillary tube, a practical method of agitation appears to be the simple expedient of moving the drop back and forth in the tube by means of controlled air pressure. Schmidt-Nielsen (1942) devised a centrifuge for sealed capillary tubes which rotates them so that liquid contained within will be thrown from one end to the other to effect mixing. The apparatus,

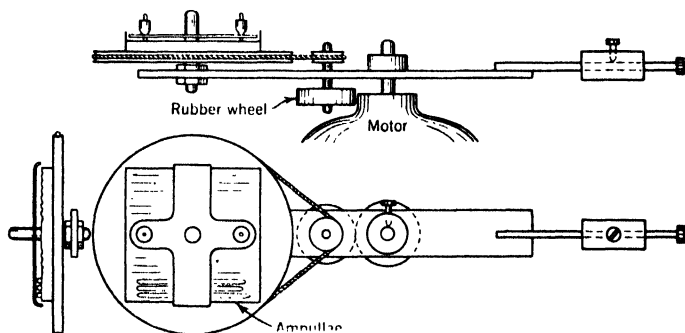



Fig. 63. Centrifuge apparatus for mixing and extracting small amounts of liquid in ampules. Length of the apparatus about 25 cm. *From Schmidt-Nielsen (1942)*

shown diagrammatically in Figure 63, is attached to the motor shaft and revolves with it. During the revolutions the plate with the ampules is slowly turned, being connected by means of a rubber band to a small rubber wheel which in turn is being driven by its frictional contact with the motor housing. The rubber wheel

is suspended in such a way that it is held against the motor housing by means of the rubber band. The tubes are turned once for about each five revolutions.

Agitation of a liquid in a capillary tube was effected by Bessey *et al.* (1946) by touching the side of the tube to a rapidly rotating nail head (page 250).

For stirring small volumes of liquid in an open shallow vessel, Kirk (1933) employed the tip of a glass needle drawn from the end of a tube in which a piece of iron was sealed. The core of an electric buzzer placed in proximity to the iron made the glass needle vibrate and stirring was thus produced. This type of stirrer is manufactured by *Microchemical Specialties Co.*

A particularly effective and convenient stirring device for small volumes in open or closed vessels is the electromagnetic "flea" of Linderstrøm-Lang and Holter (1931). The "flea" consists of a sealed glass spherical shell, about 1–2 mm. in diameter, filled with ferrum reductum; stirring is effected by an electromagnet repeatedly turned off and on by means of an interrupter. The core of the magnet is placed near the outer wall of the vessel, and the lifting and dropping of the "flea" provides the agitation. The arrangement employed in titration is shown in Figure 64. The interrupter is not shown; it is a small glass-enclosed mercury switch mounted on a pivot which is connected to a movable strip of iron in the field of the magnet. When the current is turned on, the magnetized core tips the iron strip, which tilts the mercury switch and thus breaks the current. The iron strip falls back when the core is no longer magnetized, and in so doing it brings the mercury switch back to its original position, which again completes the circuit, magnetizes the core, and starts the process over again. "Fleas" are made by blowing a small bulb in the end of a drawn-out piece of glass tubing, tapping a little ferrum reductum down into the bulb, and sealing off the neck with a microflame. The "fleas" may be cleaned by rinsing with water and covering them with fuming nitric acid. After washing well with distilled water, the "fleas" are allowed to dry on a piece of filter paper. The development of a brown stain of iron oxide on the filter paper under a "flea" indicates that it has an incomplete seal and it should be discarded. A method of testing the "fleas" suggested by Linderstrøm-Lang and Holter (1940) is to place a drop of neutral bromothymol blue solution on each one on a glass plate. Those not properly sealed

will become apparent since the acid that seeped into them during the cleaning will cause the indicator to turn yellow. The complete stirring equipment is available from *A. H. Thomas Co.* and E. Petersen.

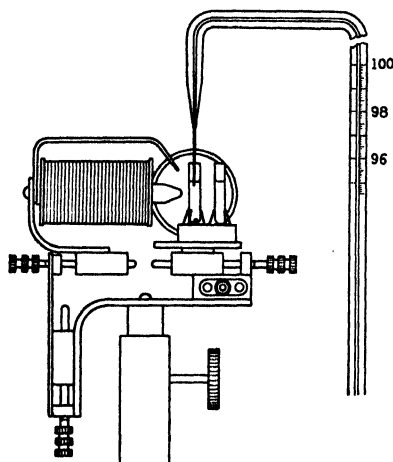



Fig. 64. Microtitration arrangement for use with magnetic "flea" stirrer. *From Linderstrøm-Lang and Holter (1940)*

Heatley, Berenblum, and Chain (1939) employed steel ball bearings, $^{1}/_{16}$ in. in diameter, given several coats of Bakelite varnish No. V-5209/2. Each coat was polymerized by stoving before applying the next, and then the balls were given a layer of paraffin by heating them to 100° in a paraffin bath. After excess paraffin was removed by rolling the bearings on hot filter paper, they were rolled in the clean, dry palm of the hand with some well-washed kaolin to enable them to be wetted by aqueous solutions. Of course these balls should not be used with liquids that might attack the coating.

E. HEATING DEVICES

A simple micro muffle furnace was described by Kirk and Bentley (1936) which, when employed with the proper rheostat, can be used for temperatures up to 1000°. The furnace is made by winding Chromel A resistance wire around a porcelain cup (2 in. inside di-

ameter, 4 in. deep), embedding in insulating material, and surrounding with iron or brass pipe.

Linderstrøm-Lang (1936) employed an incineration oven for the ashing of samples in small tubes. The oven (Fig. 65) is made of a solid copper block containing holes 18 mm. deep and about 7 mm. in diameter. Two electric heaters placed at the sides of the block enable a temperature of 440–460° to be maintained. A rheostat is used to obtain lower temperatures and to regulate the heating. The sides and bottom of the oven are insulated with asbestos. The tubes used with this oven were of quartz and had an inner diameter of 3.8 mm., an outer diameter of 6 mm., and a length of 20 mm. A solid copper block with holes drilled to accept small tubes for Kjeldahl digestions was used with gas heat by Borsook and Dubnoff (1939), as shown in Figure 66.

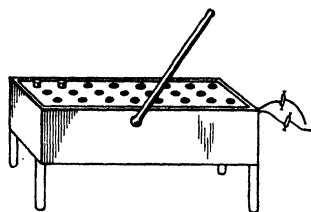



Fig. 65. Incineration oven to accommodate small tubes. *From Linderstrøm-Lang (1936)*

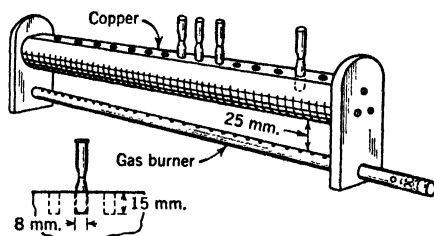



Fig. 66. Digestion rack and Kjeldahl tubes. *From Borsook and Dubnoff (1939)*

Naturally, modifications in the micro furnaces and ovens may be made, and commercially available types such as that of *Microchemical Specialties Co.* are also often suitable.

F. MOIST CHAMBERS

When working with small volumes of liquid it is necessary in certain instances to maintain a moist atmosphere around the liquid to prevent evaporation. The various forms of the moist chambers employed on the stage of a microscope have been described by Chambers and Kopac (1937). In general these are partially en-

closed chambers containing strips of wet filter paper, or a layer of water on the floor.

Holter (1945) described a large moist chamber into which the hands may be placed for various operations, and into the top of which a binocular dissecting microscope is fitted to enable observation of the material being manipulated. The humidity is kept constant through the regulation afforded by an electrically fitted hygrometer connected to a circulation pump which supplies an adjustable proportion of wet and dry air.

Holter Moist Chamber. The chamber is illustrated in Figure 67. It is made of varnished plywood; the dimensions of the box are 64 × 35 × 30 cm., not considering the bevelled surfaces on the ends of the front which contain doors 12 × 12 cm. The hands may be inserted through tight-fitting rubber cuffs attached in the openings of the doors. A slanting glass window (20 × 20 cm.) is fitted into the front of the box and is hinged so that larger objects can be

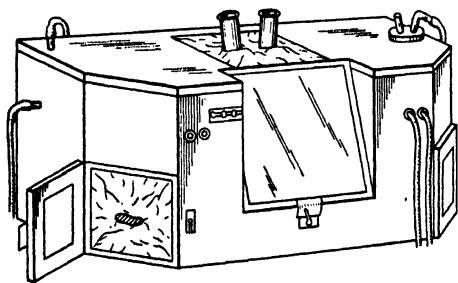



Fig. 67. Air-conditioning chamber. *From Holter (1945)*

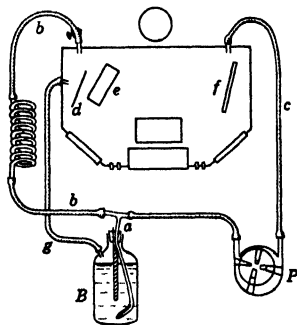



Fig. 68. Arrangement of air-conditioning apparatus. *From Holter (1945)*

placed inside. The edges of a sheet of rubber are sealed into a hole (15 × 15 cm.) cut in the top of the box; holes in the rubber fit tightly around the tubes of a binocular dissecting microscope. The sheet of rubber is rather limp and bulging so that vertical movements of the microscope will not cause it to stretch unduly. Illumination of the interior is supplied through a window in the back wall which, for some purposes, should be screened with a heat-absorbing device.

A moist atmosphere is maintained in the box by means of the

arrangement shown in Figure 68. An electric circulation pump (*P*) sends a current of air, divided by the T-tube (*a*), into the chamber. The water in the large bottle (*B*) is warmed by a lamp (about 40 watt) to a temperature 5° higher than that of the room, and the copper coil attached to the arm (*b*) is surrounded with water cooled 5° below the room temperature. The air passing into the water in (*B*) is dispersed into fine bubbles. The currents of warm and cool air enter the chamber at the same corner and the baffle (*d*) permits them to mix without allowing water droplets to be carried into the center of the box. The box contains a thermometer (*f*) and a hair hygrometer (*e*) fitted with an electrical contact at the percentage of moisture desired. By means of pinch cocks on tubes *g* and *b*, the warm air stream is first regulated so that the temperature rise in the chamber is about 1° in 15 min. when the cool air is shut off, and then sufficient cool air is admitted to compensate for this temperature rise. The contact on the hygrometer is connected through a relay to the pump which automatically stops when the desired humidity is attained and starts when it begins to fall. A thermoelectric control of the air flow through *a* or *b* could be used to achieve finer regulation, but Holter found it unnecessary for his work.

G. ELECTRODES

Linderstrøm-Lang, Palmer, and Holter Silver Electrode. A simple electrode arrangement (Fig. 69) was used by Linderstrøm-Lang, Palmer, and Holter (1935) for the micro determination of chloride by electrometric titration. The silver wire electrodes (*A*) and (*A'*) are employed as shown. *A* is fixed with a bit of picein in the side tube of the tip of the burette (*B*). Before sealing it in the side tube the wire is cleaned with a little cold dilute nitric acid, and afterward it is kept in contact with the acid silver nitrate titration solution. In this manner it need not be changed for months. *A'* requires occasional cleaning with a little nitric acid and, if necessary, fine emery cloth may be used. After titration this electrode is dried with filter paper, taking care not to touch it with the fingers. The tip of the burette may be protected from contact with *A'* by the glass cap shown on the right of Figure 69.

Sisco, Cunningham, and Kirk Glass Electrode. An open-cup glass electrode was used by Sisco, Cunningham, and Kirk (1941)

for formol titrations in the manner shown in Figure 70. The cup is made by blowing a bulb and then sucking in a depression. The

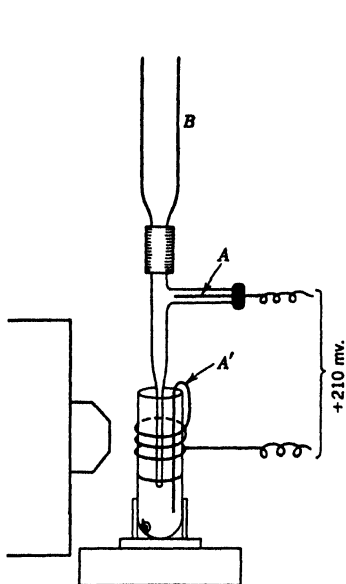



Fig. 69. Fig. 70.

Fig. 69. Silver electrodes (A and A') arranged for chloride titration with burette (B) and electromagnet on the left to enable stirring with the "flea" in the vessel (see page 282). Tip of burette may be protected from contact with A' by the glass sleeve shown on the right. *From Linderstrøm-Lang, Palmer, and Holter (1935)*

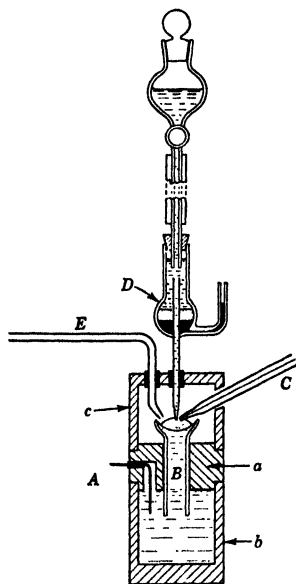



Fig. 70. Cross section of the glass electrode titration vessel. B represents an inverted glass electrode, C a burette, D a reference calomel cell, E a glass tube. A is a cup assembly, consisting of: a, a central block; b, a lower cup; c, an upper inverted cup. *From Sisco, Cunningham, and Kirk (1941)*

outside of the cup is coated with paraffin and the electrode is filled with 0.1 N hydrochloric acid saturated with quinhydrone. The chamber (A) is made of Lucite. Stirring is effected by blowing a stream of nitrogen through the tube (E) in such a fashion as to whirl the sample drop in the cup.

Claff and Swenson Glass Capillary Electrode. Glass electrodes employed for measurements of the hydrogen ion concentration of small volumes of solutions have been numerous. One of the more

KCl reservoir
Raised outlet
Ag-AgCl electrode
Corning 015 glass capillary
Lucite wafer (insulator)
Picein
Paraffin
Paraffin
Picein plug
Calomel half-cell
Liquid junction
Picein
Lucite wafer (insulator)
0.1 N HCl
Heated glass rod

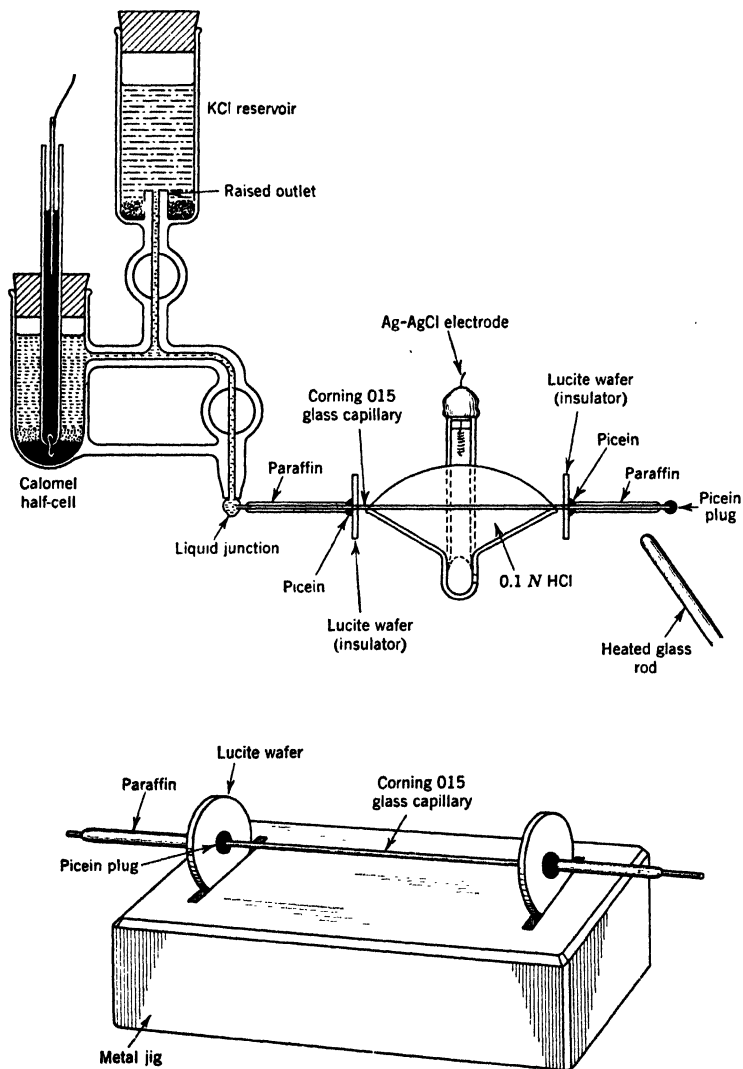



Fig. 71. Capillary glass electrode assembly.
From Claff and Swenson (1944)

recent of these is that described by Claff and Swenson (1944), which can be used for volumes as little as 5 μl. and is capable of reproducibility in measurement of ±0.02 *p*H units. The apparatus is indicated in Figure 71. The glass electrode is a capillary tube of Corning No. 015 glass 75 mm. long, attached to two Lucite wafers with picein as shown. The capillary assembly is fitted in a jig so that the wafers will always be a fixed distance apart. Starting 4 mm. from each end, the capillary is brushed with hot paraffin up to the wafers. One end of the capillary tube is plugged with picein and the open end dips into a drop of the saturated potassium chloride solution from the calomel half cell. The portion of the capillary tube between the wafers is immersed in 0.1 *N* hydrochloric acid contained in a flattened Pyrex funnel. The funnel is filled to capacity, surface tension preventing the solution from overflowing. The stem on the funnel is bent upward to form a silver-silver chloride electrode, and the funnel unit is mounted on an insulated standard so that it can be raised and lowered or moved horizontally. The entire assembly is electrically shielded, and shielded leads connecting to the *p*H meter are used. The capillary tubes are cleaned by sucking through them in the following order: Keego cleaner (*J. B. Ford Co.*) 0.1 *N* hydrochloric acid, alcohol, distilled water, and, if blood is to be used, 0.2% potassium oxalate. The tubes are then dried by drawing air through them. The tubes are stored in 0.1 *N* hydrochloric acid when not in use.

Pickford Sealed-In Capillary Glass Electrode. A permanently mounted capillary glass electrode was described by Pickford (1937). The capillary, made of Corning No. 015 glass, is sealed into the apparatus as shown in Figure 72. The three-way stopcock is of the type developed by Stadie, O'Brien, and Lang (1931). Of course it must be made of the same kind of glass as that used in the electrode jacket. A fine bore (1 mm. diameter) in the stopcock plug is required for filling in order to keep the volume of the sample as small as possible. The bore of the filling and connecting tube is about 0.5 mm. A 1 ml. syringe (preferably of the short insulin type) is employed to fill the electrode, using the assembly shown. The syringe may be used to obtain the sample, in which case the needle would be removed after the sample was taken. A short piece of hemocytometer tubing (3 mm. inside and 5 mm. outside diameter) is then slipped over the nozzle of the syringe and this is connected to the

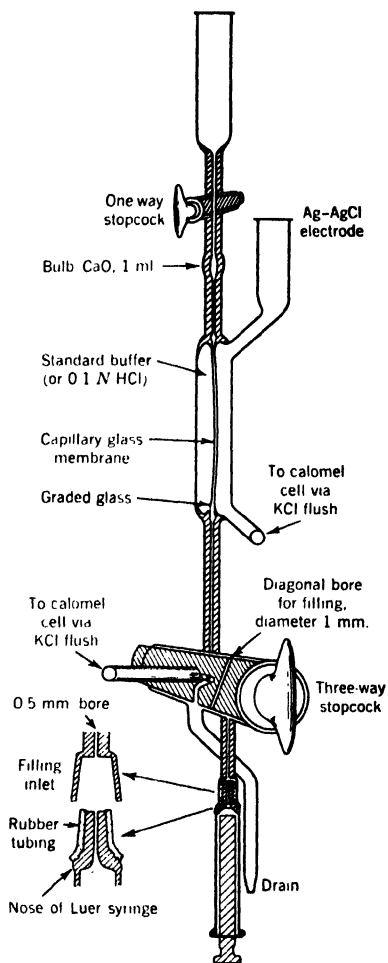



Fig. 72. Sealed-in capillary glass electrode assembly. *From Pickford (1937)*

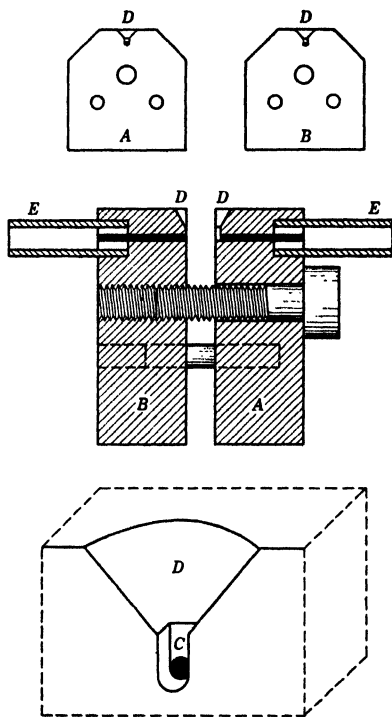



Fig. 73. Conductivity cell. The upper section shows the faces of the two blocks which make the cell (actual size), the center section, the construction, and below is an enlarged diagram of the recess which holds the fluid. *From Bayliss and Walker (1930)*

cup attached to the filling inlet. The electrode is calibrated with standard buffer, washed, and then dried with alcohol and ether. After the sample has been introduced the lower stopcock is rotated clockwise, first to flush saturated potassium chloride through the groove and right-angle bore (the position shown in Fig. 72) and

then to make liquid junction between the sample and the calomel half cell. After use the electrode is washed with dilute salt solution and kept filled with distilled water. The outer jacket is filled with 0.1 N hydrochloric acid. Pickford used a Pliotron tube amplifier with the apparatus, and the electrodes were made by *Macalister Bicknell Co.*

H. CONDUCTIVITY APPARATUS

The Bayliss-Walker Cell. A conductivity cell was designed by Bayliss and Walker (1930) for measurements on as little as 0.5 μl. of liquid (Fig. 73). Two blocks of vulcanite (A, B) are shaped and drilled as shown. Two small holes are drilled near the upper edges and in one block an enlargement is made to form a recess (C) 0.5 mm. deep, 0.75 mm. wide, and 1 mm. long in the face of the block. In each of the small holes platinum wire (0.3 mm. diameter) is sealed with sealing wax, taking care that the end of the wire is flush with the bottom of the recess in the one case and with the face of the block in the other. Glass tubes (E) are sealed into the blocks with sealing wax, as shown, and when filled with mercury enable electrical contact to be established. The blocks faced to fit perfectly against each other are located by two pins and held firmly together by a thumb screw so that a small cavity having a platinum electrode in each face is formed. A conical hole (D) is made in the face of the block nearest the cavity. The recess is lined with sealing wax, which has to be replaced from time to time as the surface deteriorates. Each electrode is coated with platinum black by covering with a drop of 2% platinum chloride, dipping a wire into the drop, applying 3 volts to the circuit, and reversing the polarity every 10 sec. for 2–3 min. It is necessary to keep the cell in distilled water, drying it only before use, in order to prevent a drift in the resistance during measurements. It is also necessary to reblack the electrodes every week or so.

The cell is filled through the conical opening with a fine pipette, using a low-power microscope to aid in the operation. The pipettes are made of small-bore glass tubing drawn out for about 10 cm. to a diameter of around 0.2 mm. or a little less. Each is cut at points 20–30 mm. from the beginning of the constriction where the diameter begins to be uniform, and the center capillary is discarded. The ends

of the pipettes thus formed are bent at right angles about 5 mm. from the small end. Care must be taken that the ends are cleanly and squarely cut. A mercury leveling bulb connected to the pipette with rubber tubing may be used for filling and emptying. Small air bubbles sometimes cling to the cell walls or to the electrodes when the cell is filled. This difficulty is overcome as a rule by withdrawing the fluid into the pipette and refilling the cell more slowly.

The circuit used by Bayliss and Walker was the standard Kohlrausch bridge fed from a 1000 cycle audiofrequency generator. The null point was determined with head phones in the usual way. Because of the small size of the electrodes and the necessity of drying them, the null point tends to be flat. A 0.01 μF. condenser placed across the standard resistance makes the null point sharp. The practice is to adjust the resistance until minimum sound intensity is obtained with the slide wire at midpoint, or until a sharp increase in intensity occurs symmetrically on each side of it.

I. BALANCES

The development of the instrumentation for the weighing of very small amounts has been thoroughly reviewed by Gorbach (1936). The commercial balances, including the torsion balances of *Roller Smith Co.*, which are sensitive down to about 2 μg., require no comment here. The quartz fiber balances, which are considerably more sensitive, are particularly useful in histochemical work and these will be considered in detail.*

Quartz Fiber Balance. Lowry (1941) designed a simple and serviceable quartz fiber balance that can handle a maximum load of 200–300 μg., and that has a sensitivity of about 0.03 μg. and a reproducibility of 0.1 μg. The functioning of the instrument (Fig. 74) depends on the measurement of the bending of a horizontal hollow quartz fiber when a weight is attached to its free end. The fiber (*A*), about 20 cm. long, is drawn from narrow quartz tubing. One end of the fiber is fused at *B* to a low tripod (*C*) made of 1–2 mm. quartz rod. Instead of fusing the fiber to a quartz tripod, the end can be inserted into a short Pyrex sleeve (having a lumen just

*** Since this writing a new quartz fiber balance has been described by Kirk *et al.*; a "Cartesian-diver" balance has been announced by Zeuthen (Bibliography Appendix, Refs. 39, 49, and 54.)**

large enough to hold it) fused to a Pyrex tripod. This enables easier replacement of fibers. The Pyrex sleeve should lean toward the front of the instrument at an angle of a little less than 90° to the plane of the tripod. The free end of the fiber (*D*) is bent into a tiny V in a plane at right angles to the fiber axis. Without a load, the free end of the fiber should be 12–15 cm. above the tripod. The tripod is mounted inside a metal cylinder (*E*), a gallon tin can will do or a smaller instrument can be made to fit into a smaller can. The open front of the cylinder is fitted with a removable glass plate (*F*).

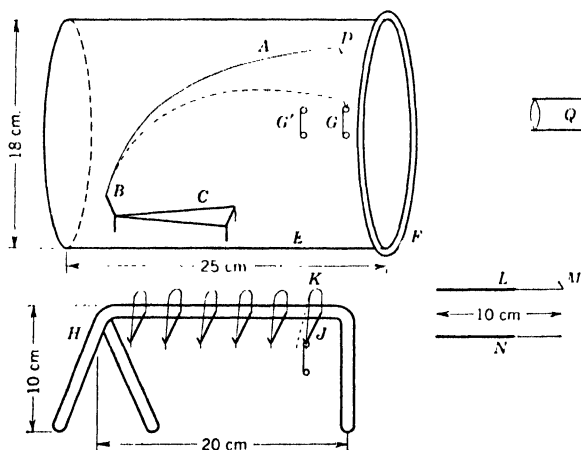



Fig. 74. Quartz-fiber balance.
From Lowry (1941)

The tripod is fixed in place with DeKhotinsky cement, and the cylinder is mounted rigidly on a heavy wooden block. A cathetometer (*Q*), reading to 0.01 mm., is used to observe the positions of an arbitrary point on the fiber tip when weights are applied. Illumination of the interior of the cylinder can be enhanced by removing the back end of the can and replacing it with a plate of glass. Electrostatic shielding can be increased by lining the inside surfaces of the glass plates with metal foil from the center of which strips have been cut to act as windows. For less accurate measurements without a cathetometer, a narrow ribbon of graph paper running down the center of the front window can serve for the indication of the displacement of the end of the fiber.

The samples to be weighed are held in quartz fiber hooks (*G*) 1 cm. long with loops at each end 2 mm. in diameter. These are made from 3–4 cm. lengths of solid fiber weighing about 30 μg./cm. One end of a piece of the fiber is held in a small oxygen flame so that the force of the flame bends the tip as it softens. By proper manipulation a complete circle can be made. The weight is now adjusted by clipping off the straight end with a scissors until the desired deflection of the balance is obtained with it. When a number of these hooks have been brought to the same weight within a few mm. deflection, the second loop is made in each one. The hooks are stored on a glass rack (*H*) which has a series of pegs (*J*) 0.5 mm. in diameter projecting from the large horizontal tube at 3 cm. intervals. The fine glass springs (*K*) prevent hooks from falling or blowing off. The hooks are handled by a glass rod (*L*) about 1 mm. in diameter drawn out at the end to 0.2 mm. A 5 mm. bend (*M*) is made in the end of this rod to slip into the loop for transfer. During attachment or removal of hooks at the end of the fiber, a straight rod (*N*) is used to hold the fiber.

After the case has been closed for 1.5–2 min., readings may be taken; successive observations have been found to agree to 0.03 mm. One hook is kept as a standard weight. Calibration of the balance is carried out by accurately pipetting 3–10 μl. of standard salt solution into the lower loop of a hook (if 1–2 μl. of distilled water is placed in the loop first, the transfer of the salt solution is easier), drying the solution, and observing the deflection given by the known weight of salt. Deflections given by various weights are plotted to form a calibration curve.

In order to obtain the dry weight of microtome sections of tissue, Lowry places a 3–5 μl. drop of water in the lower loop of a weighed hook with a fine-tipped pipette, and then places the section in the drop with a fine rod. Hooks with sections are put on the rack, dried at 100° for 30 min., and reweighed. For the measurement of neutral fat, the hooks with the dried sections may be kept in ethyl or petroleum ether for 30 min., redried in the oven, and reweighed.

Quartz Torsion Balance. A quartz torsion balance was devised by Lowry (1944) that has a capacity of 50–100 mg. and a sensitivity of ±0.1 μg. The instrument is shown diagrammatically in Figure 75. The beam (*A*) is a quartz tube 25 cm. long and about 1 mm. in diameter suspended between the horizontal quartz fibers (*C*).

The quartz stand (B) supports these fibers. Fine quartz loops in the ends of the beam hold quartz hooks (E) from which the aluminum foil pans (D) are suspended. The two arms of the beam need not be of exactly the same length. The feet (G) of the standard are sealed to the floor of a balance case with DeKhotinsky cement. It is convenient to employ the usual mechanism in analytical balances that lifts the beam when loads are added to, or removed from, the pans. Electrostatic shielding is achieved by lining the inside of the balance case with metal foil in which windows (H) are cut. In addition, the members of the balance are metalized by coating them with a 5% solution of chloroplatinic acid in alcohol and, after drying, heating with a "cool" flame to effect conversion to metallic platinum. Particular care is required to avoid overheating the fine quartz suspensions. A strip of aluminum foil is used to ground the instrument to the case.

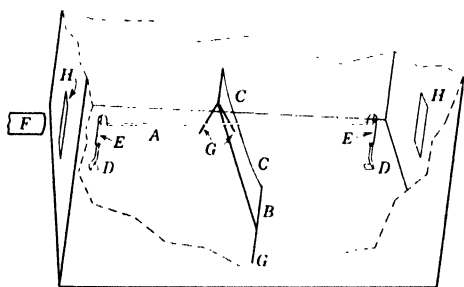



Fig. 75. Quartz torsion balance.
From Lowry (1944)

After the case has been closed for at least 1 min., measurement of the displacement of one end of the beam produced by the load placed on a pan is made with a cathetometer (F) reading to ± 0.01 mm. In a particular balance constructed by Lowry, a load of 10.8 μg. produced a displacement of 1.00 mm. The cathetometer may be focused on any convenient landmark on one end of the beam. An illuminated piece of white paper outside the opposite end of the balance case furnishes a background that facilitates the measurement.

Calibration of the balance is carried out by cutting 5–10 cm. of fine wire, weighing 1.5–2 mg., into ten nearly equal lengths, weigh-

ing the ten pieces together on a microbalance, and then observing the displacement given by each piece placed separately on a pan. The sum of the individual displacements divided by the total weight gives the sensitivity. The process must be repeated on the other pan if both arms are to be calibrated. The balance will accommodate larger weights if a tare is used as a counterbalance.

II. COLORIMETRIC TECHNIQUES

A. CAPILLARY TUBE TECHNIQUE

During the course of their classical investigations dealing with the composition of glomerular urine, Richards and his group at the University of Pennsylvania developed a simple and clever technique of capillary tube colorimetry which enabled them to carry out analyses on less than 1 μl. liquid with an accuracy comparable to that of macro procedures. The chief problem, as stated by Richards *et al.* (1933), was "to introduce the minute amount of fluid to be analyzed into a capillary tube without evaporation or contamination, to dilute it quantitatively with water if necessary, to introduce into the same capillary in quantitatively accurate proportions and without mixing the one or more reagents required for production of color, to effect mixture of the fluids in the capillary tube at a given moment, and to compare the resulting color with those developed in standard solutions treated simultaneously in identical or equivalent fashion."

The recent introduction of microcuvettes for the colorimetry of small volumes of liquid in photoelectric apparatus (page 216) will very largely displace capillary tube colorimetry because of the obvious advantages of greater objectivity and accuracy of the analyses, and the greater ease of manipulation in most cases. However, the capillary tube technique and methods are included here because there are instances in which the equipment for the cuvette methods is not available, or the volumes to be handled are still too small to permit the use of cuvettes, even of the micro variety. Furthermore, some of the capillary tube methods might be adapted to cuvette colorimetry when the equipment for the latter is available, and in that case the assembly of the methodology of the former would also be useful.

1. Apparatus

Capillary Tubes. For blood collections, plasma protein precipitations, and for the making of pipettes, capillary tubing having an outside diameter of 0.8 mm. and an inside diameter of 0.6–0.7 mm. was employed by Richards *et al.* (1933). The capillaries in which reactions were produced and colors developed were 0.5 mm. outside diameter and 0.35 mm. inside. These smaller tubes must have very uniform bores and hence it is necessary that they be drawn mechanically.

Pipettes. The pipettes are drawn from the larger capillary tubing. Their slender tips should have an outside diameter of about 50 μ; the over-all length should be about 10 cm. Liquid is drawn up and expelled in the pipettes by means of an attached piece of rubber tubing through which suction or pressure may be applied.

Microscope. A binocular microscope giving about fifteen fold magnification with an optical field of about 1 cm. in diameter is recommended. For the microscopic measurements a micrometer disc is placed in one of the oculars or the disc is cemented to the glass stage of the microscope. The disc should have a 10 mm. scale divided in 0.1 mm. In order to reduce the chance of evaporation of fluids, the glass stage, with the exception of the circle visible in the optical field, is covered with wet filter paper.

Water Manipulator. For the introduction and movement of columns of fluid in the capillary tubes, controllable suction or pressure must be applied at one end. A small syringe having a piston 3 mm. in diameter moved by a micrometer screw serves this purpose. The tip of the syringe is connected by rubber tubing with a short glass or metal tube drawn out at one end to a tip small enough to enter the capillary tube. The syringe, rubber tube, and tip are filled with colored water, care being taken to exclude air bubbles, and mounted on a level with the microscope stage. When water is forced out of the tip into the capillary tube, a water seal is formed which permits the movement of water into or out of the capillary. In this fashion columns of liquid may be introduced into the capillary tube from the other end and their movements can be easily controlled.

Other Accessories. A small centrifuge is required that will hold the capillary tubes. A piece of unglazed milk glass (35 cm. $\times$ 35 cm. $\times$ 4 mm.), two desk lamps fitted with 100 watt bulbs, and a sus-

pended lamp equipped with a 150 watt bulb and Daylight glass filter are also needed.

2. Manipulations

1. Connect a length of capillary tubing to the water manipulator, and fix the tube on the stage of the microscope so that it is parallel to, and lying on, the micrometer scale with its open end near the edge of the optical field farthest from the manipulator.

2. Force water from the manipulator into the tube until half of its length is filled.

3. Bring the tip of a pipette filled with the solution into the optical field and insert it into the open end of the capillary tube. Carefully blow the liquid out of the pipette, at the same time drawing it into the tube by turning the piston screw of the water manipulator. The volume of liquid introduced is determined by the length of the column as measured on the micrometer scale visible through the tube. When the appropriate amount of the solution has been introduced move the column inward so that its distal meniscus is near the center of the field.

4. In the same manner introduce columns of reagents, and, when these have been added, break off the portion of the tube containing all the columns (about 3–4 cm. long) and seal both ends quickly in a minute gas flame. Set aside in a horizontal position. When breaking off the tube, caution is required to avoid including any portion of the tube which has been wetted with the manipulator water (a diamond point is useful for cutting the tubes at the proper place) and when the ends are sealed, care must be exercised to avoid heating adjacent liquid columns.

5. In order to mix the solutions, briefly centrifuge the sealed tubes to bring the separated columns together, invert, and again centrifuge. Then repeat the inversion and centrifugation. (This may be simplified, see page 178.)

6. When it is necessary to heat the mixture, place the tubes in a hot water bath.

7. Since it is essential that color comparisons be made with tubes of the same diameter, the tubes to be compared should be obtained from the same original length of uniform-bore tubing. When many tubes are to be compared, the various original lengths of tubing required may not have the same diameters. In this case break a single

30 cm. length of uniform tubing into 2 cm. pieces. Transfer the colored solutions to these pieces by breaking off the sealed ends of the tubes, inserting one end of a tube into small rubber tubing held in the mouth, and placing the other end in contact with the piece into which the solution is to be transferred. Apply gentle pressure to effect the transfer and quickly seal the ends of the tube with plasticine, taking care to avoid contact between the plasticine and the solution in the tube. In order to prevent blowing the liquid from one tube right through the other, the two tubes should be held in the position of a wide-angled V during the transfer.

8. For the comparison of blue colors, place two desk lamps fitted with 100 watt bulbs side by side about 6 in. over the milk glass plate. The use of two lamps prevents shadows. For colors at the red end of the spectrum suspend a 150 watt lamp provided with color filters over the plate. Place the standard tubes, one at a time, beside the unknown on the plate for comparison. It is sometimes helpful to cover the tubes with a piece of white paper in which a rectangular window has been cut so that the visible columns are of the same length.

NOTE: In some cases it has been found that the intensity of the color developed when minute quantities of test solution and reagent are mixed in capillary tubes is not the same as when the liquids are mixed in the same proportions in macro quantities. However, when this difference does not exist, it is obviously less laborious to prepare the series of standard color mixtures in macro volumes and transfer them to capillary tubes.

3. Methods

PREPARATION OF PROTEIN-FREE SUPERNATANTS

The following procedures were employed by Richards' group (1933) for frog plasma. The proportion of plasma to precipitating reagent and the final dilution may be varied to suit the particular kind of blood used. In general it is best to keep the dilution of plasma as low as possible for capillary tube colorimetry.

Tungstic Acid Supernatants

1. Collect the blood directly in one of the micropipettes. A few grains of dry sodium oxalate may be placed in the pipette in advance.

2. Seal off the larger end of the pipette in a minute gas flame and centrifuge at once.

3. Cut the tube a little above the juncture of the cells and plasma, let the plasma flow back from the cut end by gravity, and then seal both ends of the tube taking care not to heat the plasma.

4. Attach a large capillary tube (0.6 mm. inside diameter) to the water manipulator and fix it on the stage of the microscope. Draw back 5 mm. from the end of a column of $^{2}/_{15}$ N sulfuric acid 5.0 mm. long.

5. Introduce a 4 mm. column of plasma and add 10% sodium tungstate to it until the column becomes 5 mm. long.

6. The two columns now in the tube are made to oscillate back and forth several times by means of the water manipulator in order to effect thorough mixing of the plasma and tungstate.

7. Break off the distal part of the tube and seal the ends. The end nearest the acid is sealed last and held in the flame long enough to make a small bulb.

8. Centrifuge the tube, bulb end down. Reverse and recentrifuge at least six times for complete precipitation. The final centrifugation should be thorough and the material should be left in the narrow end of the tube.

9. Break the tube about 5 mm. above the surface of the fluid and draw off the protein-free liquid into a pipette. Should a zone of haziness exist between the clear fluid and the precipitate, too much oxalate was used.

Trichloroacetic Acid Supernatants

1. For frog plasma phosphates a 4 mm. column of plasma is placed in a larger capillary tube followed by 1 mm. of 90% trichloroacetic acid (by weight). Seal the tube and centrifuge with the plasma end down.

2. Immerse in hot water for a moment and centrifuge several times inverting the tube each time.

3. Should the supernatant fluid be turbid, separate from the precipitate by cutting the tube, draw into another pipette, seal the large end, and centrifuge at high speed.

4. Separate the sediment by cutting off the tube. The protein-free liquid is ready for transfer to a mixing capillary for color development.

Zinc Sulfate-Sodium Hydroxide Supernatants

1. For frog plasma chlorides a 3.0 mm. column of plasma is drawn in 1 cm. from the end of a 10–12 cm. capillary tube followed by separate columns of 6.0 mm. of 0.1 *N* sodium hydroxide and 2.1 cm. of 0.64% zinc sulfate ($ZnSO_4.7H_2O$, freed from excess acid by three recrystallizations from water).
2. Draw in the columns so that at least 2 cm. from the end of the tube is empty, break off the portion of the tube containing the liquids, and seal both ends in a flame.
3. Place in the centrifuge with the plasma uppermost and mix the liquids by four centrifugations.
4. Immerse the tube for 30 sec. in water at 90–95° and centrifuge once for 5 min.
5. Cut off the tube above the fluid and then cut off the part containing the protein precipitate. The protein-free fluid is then ready for analysis.

CHLORIDE

By the use of *sym*-diphenylcarbazide (Cazeneuve reagent) Westfall, Findley, and Richards (1934) increased the sensitivity of Isaac's (1922) method for the determination of chloride and then adapted it to capillary tube colorimetry. Their procedure allows chloride determination in a fraction of a μl. of liquid containing 1 μg or less of sodium chloride with an average error of under 3.0%. The principle of the method is that dry silver chromate will react with chloride to precipitate silver chloride and leave the chromate ion, which can be estimated by its yellow color. However, a much more intense purple-red color will develop in the presence of diphenylcarbazide. For other methods see pages 224 and 281.

Westfall, Findley, and Richards Method for Chlorides

SPECIAL REAGENTS

Potassium Chromate Standards. Dissolve 3.321 g. of pure dry potassium chromate (corresponding to 2.00 g. sodium chloride) in 1 l. distilled water. Dilute to prepare standard solns. in the range 10–70 milligram per cent sodium chloride at 2.5 milligram per cent intervals.

Powdered Silver Chromate. Add slowly 200 ml. 5.5% potassium chromate to 100 ml. boiling 10% silver nitrate soln. Add drops of the chromate soln. until a slight excess is present as indicated by a yellow color. Cool, wash the precipitate with water, and air-dry on a Buchner funnel.

Diphenylcarbazide Reagent. Dissolve 0.5 g. *sym*-diphenylcarbazide (*Eastman Kodak Co.*) in 70 ml. 95% alcohol; add 25 ml. glacial acetic acid, and make up to 100 ml. with distilled water. This reagent is stable at 20° for only 3 hr.

PROCEDURE

1. Since it will be necessary to measure columns of liquid longer than the diameter of the optical field of the microscope, mount a 15 cm. steel rule, graduated in 0.5 mm., on the microscope stage.

2. Fill a 10–12 cm. capillary tube (0.35 mm. inside diameter) to nearly half its length with water from the water manipulator, and adjust the tube so that its open end is in the optical field over the zero mark of the stage micrometer and adjacent to the zero of the steel rule.

3. Introduce a 2–3 mm. column of zinc sulfate-sodium hydroxide supernatant (page 200) or other fluid to be analyzed and measure its length accurately, then introduce just nine times as much distilled water. If the concentration of the unknown corresponds to less than 0.1% sodium chloride, less water will be required; if higher than 0.7%, more water must be used.

4. Draw the liquid in 2 cm. from the open end, break off the portion of the tube containing the added liquids, seal the ends in a flame, and mix well by eight brief centrifugations, reversing the tube after each one.

5. Seal one end of a larger capillary tube (0.6 mm. inside diameter), place a few grains of dry silver chromate in it and tap the tube to get the material down to the closed end. Take care that none of the substance is left near the open end; cut off a little of the tube if necessary.

6. Cut off the end of the first (smaller) tube above the column of liquid, insert the open end into the silver chromate tube so that it projects into it for about 1 cm. and fasten the two tubes together with a ring of DeKhotinsky cement.

7. Centrifuge with the larger tube down for a moment so that

the liquid in the small tube is forced into contact with the silver chromate in the larger tube.

8. Warm the cement, withdraw and discard the smaller tube, cut away any of the larger tube to which the cement is adhering, and seal the open end in a flame.

9. Drive the silver chromate back and forth through the liquid by eight successive centrifugations. Continue the last centrifugation for 5 min.

10. Make a pipette from the smaller capillary tubing and draw the supernatant fluid into it.

11. Seal the larger end of the pipette, and force the liquid into this end by centrifuging for 5 min.

12. Examine the tube under the microscope (magnification 50×) to be sure the fluid is free of silver chromate particles. If not, transfer to another pipette and centrifuge again.

13. Place a new piece of the smaller tubing 10–20 cm. on the microscope stage with one end in the optical field and the other connected to the water manipulator.

14. Introduce a 2.0 mm. column of the chromate liquid obtained in step 12, and after measuring its length accurately draw it in at least 2 cm. from the end of the tube.

15. Introduce a column of the diphenylcarbazide reagent fourteen times the length of the chromate fluid, draw both columns in 2 cm. from the end, break off the portion of the tube containing the liquid, seal the ends, and place in the centrifuge with the chromate fluid uppermost.

16. Measure 4.2 ml. diphenylcarbazide reagent into each of three test tubes. Start the centrifuge containing the capillary tube, and as quickly as possible measure into the test tubes 0.3 ml. of each of three standard chromate solns. covering the range of concentration within which the unknown lies.

17. Centrifuge the capillary tube eight times, inverting it after each centrifugation.

18. Fill a piece of capillary tubing at least 3 cm. long, having the same diameter as that containing the unknown, from each test tube and seal the ends with plasticine.

19. Compare the colors of the unknown and standards on a milk glass plate under Daylite electric bulbs.

20. The result obtained gives a first approximation of the chloride

concentration of the unknown. It may be necessary to repeat once or twice with fresh portions of chromate supernatant in order that the final color comparison may be made to standards differing from one another by the equivalent of 1.25 milligram per cent sodium chloride. Smaller differences must be estimated.

SODIUM

A method for the determination of sodium in samples containing as little as 0.3 μg., *e.g.*, 0.2 μl. urine, with an average error of about 3%, was described by Bott (1943). Since this method employs a 6 ml. volume for the development of color, it is obvious that smaller quantities might be measured if the colorimetry were performed on smaller volumes. The method depends on the precipitation of the sodium as sodium zinc uranium acetate, and the measurement of the zinc in a solution of the salt by means of the red color it produces with diphenylthiocarbazone. This principle was used by Deckert (1935) for the determination of zinc, and the technique of Bott could be adapted to the measurement of small quantities of zinc. For other methods see pages 265 and 270.

Bott Method for Sodium

SPECIAL REAGENTS

Water. The water employed in preparing all of the reagents is redistilled from an all-Pyrex still.

20% Trichlorocetic Acid. Made from acid that has been redistilled from an all-Pyrex still.

95% Alcohol.

Ether. Redistilled.

0.01 N Sodium Hydroxide. Carbonate free.

Zinc Uranium Acetate Reagent. According to Butler and Tuthill (1931): Prepare a soln. of 80 g. sodium-free uranium acetate, $UO_2(C_2H_8O_2)_2.2H_2O$, and 48 g. or 46 ml. 30% (by vol.) acetic acid in water to make a total of 520 g. Prepare a second soln. of 220 g. zinc acetate, $Zn(C_2H_3O_2)_2.2H_2O$, and 24 g. or 23 ml. of the 30% acetic acid in water to make a total of 520 g. Cover and warm both solns. on a steam bath until, with stirring, soln. is complete. Mix while hot, let stand 24 hr., and if no yellow precipitate appears add 0.2 g. precipitated uranyl zinc sodium acetate in order

to saturate the soln. Shake well and filter through quantitative paper before using.

Magnesium Uranium Acetate Reagent. According to Blanchetière (1923): Dissolve 100 g. uranium acetate in 60 g. glacial acetic acid and enough water to make 1 l. Dissolve 333 g. of magnesium acetate in 60 g. glacial acetic acid and enough water to make 1 l. Combine equal vol. of the two solns. Filter the reagent through quantitative filter paper before use.

Diphenylthiocarbazone solution. Prepare immediately before use by shaking 100 mg. of the compound (*Eastman Kodak*) in 5 ml. of the sodium hydroxide soln. for 3 min. in a glass-stoppered vessel the ground surfaces of which are thinly coated with paraffin. Filter off the excess reagent on quantitative filter paper which has been washed in redistilled water and dried before use. Dilute 1 vol. of the filtrate with 4 vol. of the sodium hydroxide soln. A considerable variation in the quality of the compound from one lot to the next has been observed.

Zinc Standards. Prepare pure sodium zinc uranium acetate by precipitating the sodium of pure sodium chloride with the zinc uranium acetate reagent. Dissolve 0.235 g. of the triple salt in redistilled water and make up to 1 l. This stock soln. contains 1 mg. zinc per 100 ml. and it will keep for years in a Pyrex bottle in the dark. Frequently prepare dilute standards containing from 10 to 70 microgram per cent of zinc by diluting the stock soln. with redistilled water.

PREPARATION OF FILTERS

1. Cut 3 cm. lengths of capillary tubing, 0.6 mm. internal diameter. Cut ends squarely or the funnel openings to be made later will be off center.

2. Partially seal one end of each piece of tubing by twirling in a microflame. Use a microscope to observe the result. The opening should be funnel shaped, about 0.1 mm. at the top and less at the bottom (Fig. 76).

3. Prepare paper pulp by teasing apart the filter paper in redistilled water and drying at 105°. Bits teased off the dried pulp are placed in the filter tubes and pushed down into the funnel end by a thin capillary tube about 6 cm. long sealed at one end. Pack each bit

of pulp separately using, alternately, the open and sealed ends of the thin capillary tube in order to obtain a filter well packed on the sides and in the center. Make the packed filters 0.6–0.8 mm. thick, and see that no spaces are present around the filter mat.

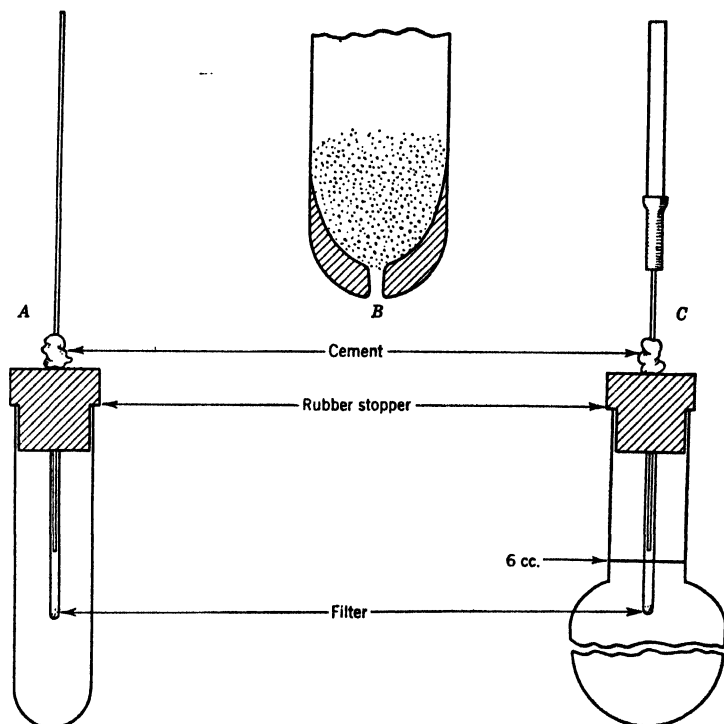



Fig. 76. Apparatus for determination of sodium. *A* and *C* are approximately actual size. *B* is an enlargement (×20) of the end of a filter tube. *From Bott (1943)*

4. Wash and test the filters by filling the tubes, with the aid of a syringe and adapter, with redistilled water. Place them in a round-bottomed centrifuge tube fitted with a mat of clean dry filter paper on the bottom, centrifuge for about 1 min., examine the tubes, and discard any which have not drained completely. Dry the filter tubes at 105° in a clean vessel and store in covered weighing bottles kept in a dust-free container.

PROCEDURE

1. As in the procedure for chloride (page 201), mount a 15 cm. length of capillary tubing (0.35 mm. inside diameter, and check the outside diameter with a stage micrometer—it should be just 0.5 mm.) on the microscope stage and fix a 15 cm. steel rule beside it so that the zero on the rule is opposite the 35 mark on the micrometer scale.
2. To one end of the tubing attach a water manipulator and place the other end over the 30 or 40 mark of the stage micrometer so that a 0.2–0.4 μl. sample will be in the center of the optical field.
3. Introduce a 2–4 mm. column of sample and draw it into the tube just far enough to give a fully curved meniscus. Measure the length of the sample column and pull it in about 5 mm.
4. Depending on the size of the sample, introduce rapidly from a rather coarse capillary pipette, just filled with freshly filtered reagent, a 30–40 mm. column of zinc uranium acetate reagent. Measurement of the reagent column need not be precise but do not move the column back and forth, since evaporation of the liquid will give high results. Draw the column in about 10 mm.
5. Cut off the portion of the tube containing the liquids, seal both ends in a microflame without heating the liquids, centrifuge and invert the tube ten times, allow to stand at room temperature for 10 min., and then centrifuge and invert five times more. During the 10 min. intervals examine the filter under the microscope and repack it gently.
6. Examine the empty end of the precipitation capillary to make sure no crystals have remained there. Cut off the end of the tube containing the precipitate and insert it into the filter tube so that the open end is about 6 mm. above the filter mat. Seal the two tubes together with DeKhotinsky cement as shown in Figure 76, taking care to avoid heating the reagent. Insert the tubes in a small hole in a rubber stopper and fit into a test tube as in *A* of Figure 76.
7. Lower the assembly into a centrifuge cup by means of rubber-tipped forceps, and spin rapidly for about 15 sec. Examine the capillary and filter; usually a little liquid is found above the filter. Cut off the sealed end of the capillary without disturbing the assembly, centrifuge again for about 6 sec., and again inspect. No fluid should appear above the mat. Centrifuge for 2 min. more to insure complete draining.

8. Set the assembly in a wooden block. Dip the end of a clean microfunnel (about 15 μl. capacity) such as pictured at the top of *C* in Figure 76 into the magnesium uranium acetate soln. Fill the funnel completely by pinching and releasing the attached rubber tubing. Wipe off the outside, and slip the rubber tubing over the end of the small capillary. Centrifuge for 6 sec., take off the funnel, and recentrifuge for 30 sec. Now repeat the process with two funnel fillings of alcohol and two of ether. After evaporation of the ether, clean, dry, yellow crystals should be seen on the filter mat.

9. Transfer the stopper and capillaries to a larger tube calibrated to contain exactly 6 ml. (*C*, Fig. 76). Introduce a little redistilled water into the small capillary, remove the funnel, and centrifuge for a few sec.

10. Cut off the capillary about 1 cm. above the DeKhotinsky cement, and proceed as before after attaching the funnel, filling the funnel with water six to seven times. This should dissolve the precipitate and transfer the liquid completely into the tube which is then filled with water to the 6 ml. mark.

11. From a 0.1 or 0.2 ml. Mohr pipette drawn out to a fine tip, add 0.1 ml. diphenylthiocarbazone soln. to each unknown tube, and also to each of three colorimeter tubes containing 6 ml. of water and of two standards, respectively. Mix the contents of all the tubes and transfer the unknowns to colorimeter tubes. The blanks should be golden yellow, and the solns. with 10–70 microgram per cent of zinc should vary from orange to cherry-red. Make colorimetric measurements immediately. With the Evelyn colorimeter use Filter 565. The calibration curve is linear, but at least one standard should be run every time an unknown is run since variations may occur even though the reagent is prepared the same way every time. The blank should be found to be negligible, since measurements carried out on redistilled water substituted for the sample gave maximum values of only ±0.6 microgram per cent of zinc.

12. Since the inside of the very curved meniscus is used for measurement of samples, make a vol. correction by the addition of 0.005 μl. for each meniscus. From the corrected vol., calculate the final dilution (in a corrected sample of 0.2 μl. vol. the dilution is 30,000 times), and apply the following relation:

$$\begin{matrix}\text{Na concn.} \\ \text{in sample}\end{matrix} = \frac{23.00}{65.38} \times \begin{matrix}\text{Zn concn. in} \\ \text{final soln.}\end{matrix} \times \text{dilution}$$

PHOSPHATE

A colorimetric capillary tube method for inorganic phosphate was developed by Walker (1933) as an adaptation of the Kuttner (1927, 1930) modification of the Bell-Doisy phosphomolybdic acid method. Walker's procedure enables analysis of as little as 0.08 μl. of liquid containing less than 1 mμg. phosphate phosphorus with a mean error of about +0.1% and a mean deviation of about ±2.5% for solutions of known concentration. A recent discussion by Sumner (1944) of phosphomolybdic acid methods should be consulted. For other methods see pages 124, 226, and 280.

Walker Method for Phosphate

SPECIAL REAGENTS

Standard Phosphate Solution. Prepare standards in the range of 1.5 to 7.0 milligram per cent phosphorus differing from one another by 0.5 milligram per cent and below 1.5 milligram per cent by 0.1 or 0.2 milligram per cent.

Molybdic-Sulfuric Acid Reagent. To 1 vol. of 10 *N* sulfuric acid (282 ml. conc. acid, 95%, sp.gr. 1.84, to 1 l.) add 2 vol. distilled water and 1 vol. 7.5% sodium molybdate soln. Store in a brown glass-stoppered bottle.

Stannous Chloride Stock Solution. Prepare a 40% soln. in conc. hydrochloric acid and store in a brown glass-stoppered bottle. Do not use longer than one week.

Stannous Chloride Working Solution. Dilute the stock soln. 1:100 and prepare fresh each day.

PROCEDURE

1. Introduce a column of about 0.2 μl. of the trichloroacetic acid supernatant (page 199) or other phosphate soln. into a capillary tube (0.35 mm. inside diameter) followed by an equal vol. of the molybdic-sulfuric acid reagent and withdraw the two columns 3 cm. from the end of the tube.

2. Introduce a third equal column of the stannous chloride working soln. and seal the ends of the portion of the tube containing the liquids.

3. In a similar manner prepare tubes with the standard phosphate solns.

4. Centrifuge all the tubes at one time with the stannous chloride soln. up.

5. Compare the colors on a milk glass background illuminated by two lamps arranged to avoid shadows. The colors fade by about 10% during the first few min. but this change occurs equally in all of the tubes and hence need not interfere with the comparisons.

PHOSPHATASE

By an adaptation of the method of King (1932) to capillary tube colorimetry, Weil and Russell (1940) worked out a procedure for the determination of phosphatase which could be applied to less than 1 μl. plasma with an average deviation of ±3.0%. Their technique employs capillary tube procedures for the determination of the inorganic phosphate, but the enzymatic digestion procedure is based on the use of pipettes, reaction tubes, and other apparatus common to the titrimetric techniques. As in the method of Siwe (1935b) (page 226), aminonaphtholsulfonic acid is used to reduce the phosphomolybdate. For other methods see page 226.

Weil and Russell Method for Phosphatase

SPECIAL REAGENTS

Standard Phosphate Solutions. Prepare standards in the range of 0.02–1.00 μg. phosphorus/15 μl. differing from one another by 0.02 or 0.04 μg.

Molybdic-Sulfuric Acid Reagent. 5% ammonium molybdate containing 15% by vol. conc. sulfuric acid.

Aminonaphtholsulfonic Acid Solution. Dissolve 0.5 g. of the 1,2,4 acid, 30 g. sodium bisulfite, and 6 g. crystalline sodium sulfite in water by shaking, and make up to 250 ml. Filter and, if filtrate is not clear, leave overnight and again filter. Prepare fresh every 2 weeks.

Veronal Buffer, pH 9.0, with magnesium. 0.0015 *M* magnesium chloride in buffer consisting of 9.36 ml. 0.1 *M* sodium diethyl barbiturate + 0.64 ml. 0.1 *N* hydrochloric acid.

Substrate Solution. 0.1 *M* sodium-β-glycerophosphate.

10% Trichloroacetic Acid.

PROCEDURE

1. Pipette 3 μl. plasma into 21 μl. water in a reaction tube of 250 μl. capacity, and add 7 μl. Veronal buffer with magnesium and 7 μl. substrate soln. Mix with a magnetic stirring "flea" (page 179).
2. Set up a control experiment in which the substrate and buffer are placed as a separate drop on the side of the tube where it cannot touch the enzyme soln.
3. Place tubes in a rack in a desiccator containing water in the bottom and a small bottle of chloroform to produce a vapor inhibiting growth of microorganisms. The desiccator is kept at 37° and the digestion is allowed to proceed for 4 hr.
4. Stop the reaction by setting the tubes in ice water, and add 10 μl. 10% trichloroacetic acid to each.
5. Centrifuge, and pipette 15 μl. of the supernatant into another tube.
6. Add 7 μl. of the molybdic-sulfuric acid reagent to the supernatant.
7. Pipette 5 μl. aminonaphtholsulfonic acid soln. on the side of the tube as a separate drop.
8. Set up standards with 15 μl. of the known phosphate solns., following steps 5–7.
9. Mix the drops on the side of the tubes with the rest of the liquid using stirring "fleas."
10. Draw the solns. into capillary tubes of uniform lumen (inside diameter 0.65 mm., length 30 mm.) and seal the ends with Duco cement.
11. Compare the colors as in the Walker method.

REDUCING SUBSTANCES

Sumner's (1925) dinitrosalicylic acid method was adapted to capillary tube colorimetry by Walker and Reisinger (1933). In this manner, quantities of glucose of the order of 0.1 μg. in 0.2 μl. liquid (50 milligram per cent) can be determined with a maximum error of 3 milligram per cent in duplicate measurements of solutions of known concentration. For other methods see page 296.

Walker and Reisinger Method for Reducing Substances

SPECIAL REAGENTS

Standard Glucose Solutions. Prepare solns. in the range 10–100 mg./100 ml. in 5 mg. steps.

Sumner Reagent. Add 22 ml. of 10% sodium hydroxide to 10 g. crystallized phenol. Dissolve in a little water and dilute to 100 ml. Add 69 ml. of this soln. to 6.9 g. sodium bisulfite; then add a soln. containing 300 ml. 4.5% sodium hydroxide, 255 g. Rochelle salt ($KNaC_4H_4O_6.4H_2O$) and 880 ml. 1% dinitrosalicylic acid. Store in well-stoppered bottles and prepare fresh each week.

PROCEDURE

1. Introduce a 1.5–3.0 mm. column of tungstic acid supernatant (page 198) or other unknown soln. into a capillary tube (0.35 mm. inner diameter) followed by a second column (three times as long) of the reagent. Seal both ends of the tube and mix by centrifuging
2. Mix the standard solns. with reagent in test tubes employing 1 ml. glucose to 3 ml. reagent.
3. Immerse the capillary tubes and the test tubes together in boiling water for 5 min.
4. Transfer the standard color solns. to capillary tubes.
5. Compare the colors on a white background under light screened with Daylite glass.

CREATININE

Bordley, Hendrix, and Richards (1933) adapted Folin's method for the determination of creatinine to capillary tube colorimetry. As finally worked out, this adaptation enables analysis of about 0.5 μl. of liquid containing 10–30 mμg. creatinine with an error of a few per cent. For other methods see page 239.

Method of Bordley *et al.* for Creatinine

SPECIAL REAGENTS

Standard Creatinine Solutions. Prepare solns. containing 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, and 6.0 milligram per cent creatinine in 0.01 *N* hydrochloric acid. Add toluene as a preservative.

Saturated Picric Acid Solution. Prepare from pure picric acid.

10% Sodium Hydroxide. Prepare from Merck's reagent "from sodium."

Folin Reagent. Freshly prepare before use by mixing 5 vol. saturated picric acid with 1 vol. of the 10% sodium hydroxide.

PROCEDURE

1. Introduce separate columns of saturated picric acid (25 micrometer divisions), tungstic acid supernatant (page 198) or other unknown soln. (60 divisions), and 10% sodium hydroxide (5 divisions) in that order into a capillary tube (0.35 mm. inside diameter).
2. Seal off the ends of the tube and place in a closed box until the other tubes are prepared.
3. Darken the room for the following operations.
4. Prepare as rapidly as possible the standard color solns. in test tubes by adding 1 ml. Folin reagent to 2 ml. standard soln.
5. With no loss of time mix the liquids in the capillary tubes by repeated centrifugations and plan to begin color comparisons 10 min. after the first centrifugation.
6. In this 10 min. interval transfer the contents of each capillary tube and a portion of each standard mixture to pieces of capillary tubing of uniform bore, and seal the ends of each piece with plasticine.
7. Place each sealed piece of tubing in a labeled space on a milk glass plate for color comparison.
8. Compare the colors in a dark room under a 200 watt bulb equipped with a straw-colored light filter, or illuminate the milk glass plate from underneath using a straw-colored filter between the plate and the light source.

NOTE: The particular order of procedure given must be followed since Folin reagent darkens at a faster rate and more extensively in capillary tubes than it does in larger volumes in test tubes. Furthermore, this change is intensified and accelerated by daylight, which makes it imperative to protect the solutions from light. The yellow picric acid color interferes with the comparisons of the red colors developed, and hence it is necessary to use a straw-colored light filter. The color produced by 2.0 milligram per cent creatinine is about the palest which can be reliably estimated in the tubes used. The most advantageous colors are those produced in the range 2.5 to 5.0 milligram per cent.

URIC ACID

Bordley and Richards (1933) adapted Folin's (1930) method for the determination of uric acid to capillary tube colorimetry with the result that 0.03–0.5 μl. liquid containing 3–10 mμg. uric acid can be determined in solutions of known concentration with an average error of about 5%. For other methods see page 239.

Bordley and Richards Method for Uric Acid

SPECIAL REAGENTS

Standard Uric Acid Solutions. Prepare stock soln. containing 1 mg./ml. Transfer 1 g. uric acid to a 1 l. volumetric flask. Shake 0.6 g. lithium carbonate in 150 cc. water for 5 min. to dissolve, and filter. Heat the soln. to 60°, pour into the liter flask with the uric acid, and shake for 5 min. Cool under running cold tap water. Add 20 ml. 40% formalin and half fill the flask with distilled water. Add a few drops of methyl orange soln. followed by 25 ml. 1 *N* sulfuric acid added slowly and with shaking. The soln. should turn pink before the last 2–3 ml. acid is added. Dilute to vol., mix well, and store in a tightly stoppered bottle protected from light. Prepare a series of standards containing 0.6, 0.8, 1.0, 1.2, 1.4, 1.6, and 2.0 mg./100 ml.

Cyanide-Urea Solution. Dissolve 50 g. sodium cyanide in 700 ml. water, add 300 g. pure urea and, when dissolved, transfer into a 2 l. flask. Add 5–6 g. calcium oxide and shake for 4–5 min. Filter through Whatman No. 41 or similar paper. Add up to 1 g. finely divided disodium phosphate; shake and filter. The soln. may be used for at least 2 months if stored at room temperature and much longer if kept cold.

Uric Acid Reagent. Dissolve 100 g. sodium tungstate in 200 ml. water. Add slowly with stirring and cooling 20 ml. 85% phosphoric acid. Pass a slow stream of hydrogen sulfide through the soln. for 20 min. but after the first 3–4 min. add 10 ml. more of the 85% phosphoric acid. Filter through Whatman No. 41 or similar paper, refiltering the first 40 ml. Transfer the filtrate to a separatory funnel and shake for a few min. with 300 ml. alcohol. Transfer the lower layer into a previously weighted 500 ml. flask and add water to a total of 300 g. liquid. Boil a few min. to remove the

hydrogen sulfide. Add 20 ml. 85% phosphoric acid and slowly boil for 1 hr. under a reflux condenser. Decolorize with a few drops of bromine, boil off the excess bromine, and cool. To 12 g. lithium carbonate and 25 ml. phosphoric acid, add slowly 150 ml. water. Boil off the carbon dioxide and when solution is complete, cool and mix with the conc. uric acid reagent and dilute to 1 l. Keep in well-stoppered bottles protected from light.

PROCEDURE

1. Introduce into a uniform capillary tube (0.35 mm. inner diameter) a 5 mm. column of tungstic acid supernatant (page 198) or other soln. to be analyzed, 5 mm. cyanide soln., and 1 mm. uric acid reagent, keeping the three columns separated by air spaces. Seal off both ends of the portion of the tube containing the liquids.
2. In a similar manner prepare tubes with the seven standard solns.
3. Mix the liquids simultaneously in all of the tubes by centrifugation.
4. Four min. after the mixing immerse the tubes for 1 min. in boiling water.
5. Compare the colors.

UREA

Walker and Hudson (1937) adapted the capillary tube apparatus to the determination of urea by the hypobromite method. This adaptation enables the analysis of 0.3 μl. liquid containing 2 to 25 milligram per cent urea nitrogen with an average deviation from the macro method of ±3.8%. The measurement of known quantities of urea added to dialyzed horse serum could be made with an average error of 2.3%. For other methods see page 286.

Walker and Hudson Method for Urea

SPECIAL REAGENTS

Sodium Hypobromite Solution. Prepare according to Stehle (1921): In a 50 ml. Erlenmeyer flask, mix 2 ml. of a soln. containing 12.5 g. sodium bromide and 12.5 g. bromine/100 ml., with 2 ml. of a soln. containing 28 g. sodium hydroxide/100 ml. Gently

revolve the mixture in the flask for 1 min. and set aside for 30 min. before use.

PROCEDURE

1. Fix a 15 cm. length of uniform capillary tubing (0.35 mm. inner diameter) to the stage of the binocular microscope so that one end is in the optical field and attach the water manipulator to the other end.
2. Introduce a 6 mm. column of distilled water with a capillary pipette and draw it back 1 mm. from the end.
3. Introduce a 3 mm. column of tungstic acid supernatant (page 198) or other urea soln. to be analyzed, draw it in away from the end, and seal the end with plasticine.
4. Accurately measure the length of the air column between the two liquid columns with a filar micrometer temporarily substituted for the right ocular which contains a disc micrometer. Two successive readings must agree within 1 micrometer scale division (5 μ).
5. Replace the right ocular, cut off the end of the tube sealed with plasticine, move the urea column back to the end of the capillary with the water manipulator, and add 3 mm. of sodium hypobromite soln. from a blunt-tipped pipette freshly filled just before use. Should gas bubbles appear immediately upon the addition of the reagent, discard the tube, and prepare fresh reagent.
6. Move the liquid column away from the end of the tube and seal with plasticine.
7. Carefully remove the tube from the water manipulator by cutting it about 6 cm. from its end, revolve it between thumb and forefinger for a few sec. and set aside in a nearly vertical position upon a plasticine mount.
8. After 2 hr. again revolve the tube for a few sec., place on the microscope stage and accurately measure the length of the air column with the filar micrometer.
9. Run a blank determination with distilled water and subtract the increase in the length of the air column from that found above. For each milligram per cent of urea nitrogen the increase averages four scale divisions (20 μ); the increase in the blank averages five divisions. Hence a soln. containing 10 milligram per cent urea nitrogen should give an increase of 45 divisions.

HYDROGEN ION CONCENTRATION

Capillary tube colorimetry has been employed for the measurement of the hydrogen ion concentration of less than 1 μl. liquid by Montgomery (1935), who used quartz capillary tubes having an internal diameter of 4–5 mm. When comparisons of indicator colors given with protein-free buffer solutions were made, the error of measurement was less than 0.02 *p*H. However when applied to biological fluids, the indicator color may not be an accurate indication of the *p*H value. Montgomery (1935) observed that the capillary tube method gave values for blood plasma from frogs and *Necturus* which were consistently lower by an average of 0.11 *p*H than those obtained with a glass electrode, a deviation which he ascribed to the protein error of the indicator. It may be possible in some cases of this nature to apply a correction factor. For electrometric measurements see page 183.

B. CUVETTE TECHNIQUE

1. Apparatus

General. Cuvettes for the colorimetric measurement of small volumes of liquid have been designed for use with certain standard colorimeters. Zeiss cuvettes having a capacity of 0.2 ml. are made for the Pulfrich step photometer. The Evelyn photoelectric colorimeter has a micro attachment made to accommodate cells which require 0.15 ml. Adapters which enable 0.2 ml. cuvettes to be used with the Coleman Junior spectrophotometer (model 6) are obtainable from *S. Ash* (Lowry, Lopez, and Bessey, 1945). Quartz cuvettes permitting the use of volumes of 0.05 ml., or less, with a special adapter for the Beckman quartz spectrophotometer have been described by Lowry and Bessey (1946). With their adaptation measurements can be carried out on 0.05 ml. volumes from about 225 to 1050 mμ with spectral widths of no more than 3 mμ. With 0.025 ml. volumes a range of 235–935 mμ can be utilized with the 3 mμ spectral bands. The cuvettes and adapters may be obtained from *Pyrocell Manufacturing Co.**

* Since this writing a capillary absorption cell has been described by Kirk *et al.* (see Bibliography Appendix, Ref. 40; see also Ref. 42).

The degree of light absorption, and consequently the response in the eye or in a photocell, is proportional to both the concentration of the color substance and the length of the light path through the solution. Therefore, a given quantity of color substance will effect the same light absorption whether it is contained in a volume of 0.002 ml. and a 0.35 mm. light path is used (as in Richards' technique, page 195), or in a 0.060 ml. volume with a 10.5 mm. path. Of course, the greatest absorption would be obtained by employing the smallest volume with the longest light path.

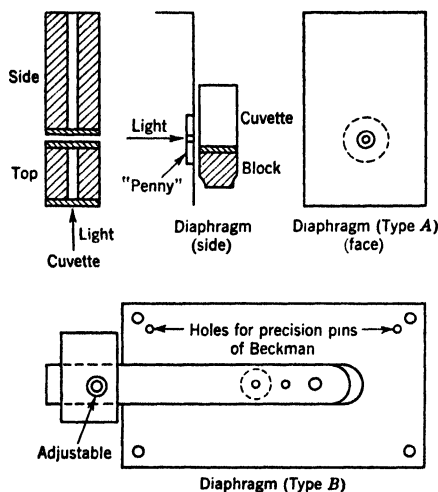



Fig. 77. Microcuvette and diaphragms.
From Lowry and Bessey (1946)

Lowry and Bessey Adaptation of Beckman Spectrophotometer to Measurements on Small Volumes. The special cuvettes used have the same 1 cm. light path as the macro variety, but the width of the chamber has been reduced to 2 mm. or less (Fig. 77). A 0.05 ml. volume of liquid will fill the cuvette to a height of about 2.5 mm. The height of the cell is 25 mm. and its outside cross-sectional dimensions are the same as those of the macro vessel. The inner cross-sectional dimensions of the macrocuvette are 10 × 10 mm. Cuvettes having an internal measurement of 1 × 10 mm. have also been used; they require 0.03 ml. liquid, but their use is more difficult.

A diaphragm is placed in front of the cuvette to obtain a light beam confined to a cross section of less than 2 × 2 mm. A beam of this size can pass through the liquid without touching the meniscus or the walls of the cuvette. The diaphragm (type A, Fig. 77) has a metal disc the size of a penny through which a 1.0 to 1.4 mm. hole is drilled about 1 mm. off center. Before the disc is fastened to the metal sheet, it is held in the opening from which the light enters the cuvette and turned until the beam passes precisely in the middle between the walls of the chamber when the cuvette is in place. The disc is soldered at this angle to the sheet of metal (about 6 × 9 cm.) so that the hole coincides with a 3–4 mm. hole in the sheet 2.5 cm. from one end. The top of the sheet is bent at a right angle to form a flange which lies on the top of the instrument. Wooden blocks are used to raise the cuvettes so that the light beam just misses the bottom of the chamber. The diaphragm is inserted and removed by loosening the bolts which hold the phototube housing. The carriage for the cuvettes should be oriented to bring the cuvettes as near the diaphragm as possible. The cuvettes are numbered and always set in the holder with the same orientation.

The type B diaphragm (Fig. 77) contains a sliding strip of brass with pinholes which can move in a channel cut in the sheet metal. The diaphragm is inserted between the cuvette carriage and the body of the instrument and the sliding strip is moved until a pinhole coincides with the center of the cuvette. The stop on the strip is then adjusted with a bolt so that the pinhole can be brought to the same position each time. The different-sized pinholes can be brought into position without disturbing the adjustment. Blocks are used to raise the cells as with the type A diaphragm. By removing the brass strip the instrument can be used with macrocuvettes without disturbing the metal sheet.

To obviate the effect of "play" in the cuvette carriage, the cells should be moved into position from the same direction. In use, the microcuvettes are left mounted in the carriage. Samples are introduced with fine-tipped pipettes, and removed by suction with fine tipped glass tubes. A macro cell may be used in the first position in the carriage for the solvent or other blank solution.*

* See Bibliography Appendix, Ref. 32.

2. Methods

CALCIUM

Sendroy (1942b) adapted iodometric reactions, previously used in titrimetric measurements of calcium, to colorimetry. Using an Evelyn macro photoelectric colorimeter, the method was applied to volumes of serum down to 20 μl. (about 2 μg. calcium). Further refinement could be obtained if the colorimetry were carried out with smaller volumes in microcuvettes. The calcium is precipitated as the oxalate; the latter is washed, dried, dissolved in acid, and reacted with an excess of ceric sulfate. The excess of ceric ion is made to liberate iodine from potassium iodide and the yellow color thus developed is measured. A precision of ±2% has been reported. An alternative method is measurement of the blue color formed when starch is added to the iodine solution. However, the many factors which influence the blue color make it a less desirable choice even though the relative color intensity of the blue is about 100 times that of the yellow (Sendroy and Alving, 1942). The method to be described was developed for serum, but it can be adapted to other fluids. A thorough study of different procedures for the determination of serum calcium was made by Sendroy (1944). For titrimetric methods see page 272.

Sendroy Method for Calcium

SPECIAL REAGENTS

Saturated Ammonium Oxalate (about 3.5%). Prepare at room temperature using analytical reagent grade of the salt.

2% Ammonium Hydroxide. Dilute 2 ml. conc. ammonium hydroxide (26% analytical reagent grade) to 100 ml.

Water-Alcohol-Ether Mixture. Mix equal vol. distilled water, absolute ethyl alcohol, or redistilled 95% alcohol, and ethyl ether (analytical reagent grade, or absolute, or redistilled U.S.P. grade).

1 N Sulfuric Acid (approx.). Dilute 27 ml. conc. acid, sp. gr. 1.84, analytical reagent grade, to 1 l.

0.2 N and 0.1 N Sulfuric Acid (approx.). Prepare from the 1 *N* soln.

0.1 N Ceric Bisulfate (approx.). Dissolve 29 g. anhydrous ceric bisulfate in 1 *N* sulfuric acid to make 500 ml. (The greater ease of solution of the bisulfate makes it preferable to the sulfate. The bisulfate is obtainable in about 92% purity from *G. Frederick Smith Chemical Co.*) Store in amber bottles and protect from light.

In preference to ceric sulfate, Kochakian and Fox (1944) have stressed the greater sharpness of the end point in titration with ammonium hexanitratocerate using setopaline C as the indicator. Prepare a 0.01 *N* soln. by dissolving 6 g. ammonium hexanitratocerate, reagent grade, in about 200 ml. 1 *N* perchloric acid and dilute to 1 l. with the acid. Do not heat during preparation, and store in a black bottle in the dark. Prepare a 0.05% setopaline C soln. by adding 50 mg. of the dye (*Eimer and Amend*) to 100 ml. distilled water and warming on a hot plate or bath. Precipitation occurs on cooling, therefore the soln. must be warmed before use and used while warm.

0.0035 N, 0.001 N, 0.0007 N, and 0.00035 N Ceric Bisulfate (approx.). Prepare these solns. just when needed from the 0.1 *N* soln. Use 0.2 *N* sulfuric acid to dilute the 0.1 *N* to 0.0035 *N*. Use 0.1 *N* sulfuric acid for dilution to the weaker concentrations. Prepare the 0.007 *N* and 0.00035 *N* solns. from the 0.0035 *N* soln. Store in amber bottles and protect from light.

Standard 0.1 N Sodium Oxalate. Dissolve 3.3498 g. sodium oxalate, analytical reagent grade, in 52 ml. 1 *N* sulfuric acid and add water to make 500 ml. Store in amber bottle; the soln. is stable for at least 6 months.

Standard 0.0005 N, 0.00025 N, and 0.0002 N Sodium Oxalate. Prepare fresh when needed from the 0.1 *N* soln. by dilution with water.

0.5% and 1% Potassium Iodide (approx.) Prepare fresh for use from analytical reagent grade of the salt. (When tested with starch, no trace of free iodine should be present.)

95% Ethyl Alcohol. Filter through two layers of ashless filter paper on a Buchner funnel.

2% and 1% Starch (Lintner Soluble). Prepare the 2% soln. in saturated sodium chloride soln. every 2 weeks by making a paste, diluting to vol. and boiling for 5–10 min. Prepare the 1% soln. fresh for use from the 2% soln. by diluting with water.

PROCEDURE

1. Mix 5 vol. distilled water with 1 vol. serum in a 12–15 ml. centrifuge tube; run in duplicate. Add 6 vol. distilled water to another centrifuge tube to be treated in a parallel manner as a standard; run in duplicate.
2. Add 1 vol. saturated ammonium oxalate to each tube, stir by tapping, cover tubes to keep out dust, and let stand at least 16 hr.
3. Centrifuge for 5 min. at 2600 R.P.M. and carefully siphon off all but about 0.2 ml. of the supernatant with an upturned capillary, the tip of which is kept immersed.
4. Wash the entire inner surface of each tube with 3 ml. 2% ammonia added slowly from a pipette moved around the top of the tube.
5. Tap the tubes until the precipitates just begin to move up; then again centrifuge and withdraw the supernatant.
6. Add 1 ml. of the water-alcohol-ether mixture; stir and mix well. Add 3 ml. more of the mixture and mix gently to keep a minimum of the precipitate in the upper portion of the liquid. Centrifuge and withdraw the supernatant.
7. Repeat the washing with the water-alcohol-ether mixture as in step 5.
8. Place the tubes in an oven at 100–110° at an angle of about 15° for 0.5–1.0 hr. to dry completely.
9. To each of the two standard tubes add different vol. of the standard oxalate soln. (See Table VI.) Add the sulfuric acid to all the tubes (see table) and heat for 5 min. in a beaker of water kept below boiling.
10. Remove tubes, let cool to room temperature and add the ceric bisulfate (Table VI). Mix well, cover the tubes, and let stand at room temperature for 30 min. or in a water bath at 70° for 10 min.
11. Transfer solns. to vessels in which color development and dilution to final vol. (Table VI) are carried out. To facilitate transfer, coat a part of the outer rim of the tube with a thin film of paraffin. Wash out tubes with 4 ml. portions of water to make the transfer quantitative.
12. Add potassium iodide (Table VI) with a minimum of agitation necessary to mix well. After 60 sec. add filtered alcohol and then water to bring to final vol.

TABLE VI

Outline of Procedures for Calcium in Serum with Evelyn Photoelectric Colorimeter (Sendroy, 1942b). The volumes are measured in milliliters.

Material	Vol. of standard $Na_2C_2O_4$ soln.	Vol. of H_2SO_4 soln.	Vol. of $Ce(HSO_4)_4$ soln.	Vol. of KI soln.	Vol. of 95% filtered alcohol	H_2O to final vol.	Filter No.[a]	Calc. constants
	0.0005 *N*	0.2 *N*	0.001 *N*	1%				
0.1 ml. serum		2.0	1.0	1.2	8.0	20	586-5[b]	$D = 200$
Standard	1.5, 0.5	2.0	1.0	1.2	8.0	20		$C = 0.05$
Reagent blank		2.5		1.2	8.0	20		
	0.00025 *N*							
0.05 ml. serum		1.0	0.5	0.6	4.0	10	586-5[b]	$D = 200$
Standard	1.5, 0.5	1.0	0.5	0.6	4.0	10		$C = 0.05$
Reagent blank		1.25		0.6	4.0	10		
	0.0002 *N*	0.1 *N*	0.00035 *N*					
0.2 ml. serum		1.0	1.0	0.6	4.0	10	586-5[b]	$D = 500$
Standard	1.0, 0.0	1.0	1.0	0.6	4.0	10		$C = 0.02$
Reagent blank		2.0		0.6	4.0	10		
		1.0 *N*	0.0007 *N*	0.5%	Starch soln., 1%			
0.05 ml. serum[c]		2.0	1.0	0.5	0.5	25	600	$D = 500$
Standard	2.0, 0.5	2.0	1.0	0.5	0.5	25		

[a] The Corning Violet Ultra No. 586 (5 mm. thick, polished glass) filter transmits light within the range of 328 to 388 (maximum 360) mμ. Evelyn (Rubicon) filters No. 400 and No. 600 transmit light within the ranges 360 to 430 (maximum 400) mμ and 580 to 635 (maximum 600) mμ, respectively.
[b] "Brighter" switch on in the Evelyn instrument.
[c] Alternative procedure when filter No. 586-5 is unavailable.

13. Prepare simultaneously reagent blanks containing sulfuric acid, potassium iodide, and alcohol in the same concentrations as in the standards and unknowns. Use these blanks to set galvanometer at 100 just before reading the standards and unknowns.

14. Read 10 ml. portions at 25 ±5° in the Evelyn colorimeter with filters indicated in the table. The yellow color may be read at any time within 1 hr. after addition of the potassium iodide. Data are given in the table for use of blue color with starch if filters for yellow color are unavailable. In the latter case, add water to about 80% of vol. after the potassium iodide has been added, add the starch slowly to the soln. at 25 ±1°, mixing by rotation, add water to vol., and read color promptly at 25 ±1°. The blue-color method is not reliable for samples of serum smaller than 0.05 ml.

Result. Since blue color readings are not reproducible from day to day, a new calibration curve must be obtained for each day's work. Galvanometer readings plotted semilogarithmically are linear functions of oxalate concentration. For calibration of yellow-color readings, the percentage transmission readings of 90, 80, 70, 60, 50, 40, 30, 25, 20, 15, and 10 correspond to the respective values of iodine, in milliequivalents per liter in the color solns., of 0.0022, 0.0048, 0.0077, 0.0111, 0.0151, 0.0200, 0.0267, 0.0308, 0.0362, 0.0433, and 0.0544. To redetermine the calibration curve, treat 0.5 to 0.7 ml. of 0.133 mM potassium iodate with 2.4 ml. of 0.085 M phosphoric acid and 1.2 ml. of 5% potassium iodide, dilute to 40 ml. with filtered 95% alcohol, and then to 100 ml. with water. Read with filter No. 586-5.

Calculate the concentration of the calcium (in milliequivalents per liter) in the unknown from:

$$\left[\frac{C-(I_2)_{s_1-s_2}}{2} - (I_2)_u\right]D$$

where s_1, s_2, and u refer to standards and unknown sample, $C = (C_2O_4^{2-})s_1 + (C_2O_4^{2-})s_2$, and $D = V/v$ where V is the volume (in ml.) at final dilution of color soln. and v is the volume (in ml.) of original sample used. The C and D values are given in the table and the (I_2) values are obtained from calibration curve data corresponding to the readings observed.

When 0.02 ml. samples of serum are used it has been found necessary to correct the yellow color readings for the effects of traces of residual serum. The correction varies with the reading and is to be subtracted from the latter: for readings of 15, 20, 30, 40, 50, and 60 subtract corrections $0^{1.5}$, 0^{2}, 0^{3}, 1^{0}, 1^{1}, and 1^{2}, respectively.

EXAMPLE: Analysis of 0.02 ml. samples of a serum gave yellow color readings of 36^{1}, 36^{1}, for the standards, 23^{1} and 55^{0}. The former were corrected to 35^{1} by subtracting 1^{0}. Values for iodine in milliequivalents per liter from the calibration curve were 0.0230 for the serum, and 0.0325 and 0.0130 for the standards. Then the calcium concentration in the original serum (in milliequivalents per liter) was:

$$\left[\left(\frac{0.0200 + 0.0325 + 0.0130}{2}\right) - 0.0230\right] \times 500 = 4.90$$

In the case of blue-color readings a semilogarithmic plot is made with milliequivalents of oxalate per liter from 0 to 0.0171 as abscissa and galvanometer readings from 10 to 100 ordinates. A straight line is drawn between the points representing the readings of the two standards, s_1 and s_2, at concentrations of 0.016 and 0.004 milliequivalent oxalate per liter. Oxalate values for serum analyses, obtained by interpolation of their galvanometer readings on this line, times D give directly milliequivalents calcium per liter in the sample.

EXAMPLE: Analysis of 0.05 ml. samples of a serum gave blue color readings of 32^{2} and 32^{3}, for the standards, 21^{1} and 49^{2}. A straight line was drawn through the two latter values located at 0.016 and 0.004 milliequivalent oxalate per liter, respectively. Interpolated values for the serum were 0.01005 and 0.01013 milliequivalents per liter. Then the average calcium concentration in the original serum was:

$$0.01008 \times 500 = 5.04 \text{ milliequivalents per liter.}$$

CHLORIDE

Colorimetric chloride methods have not been specifically adapted to histochemical work. However, the procedure of Sendroy (1939b, 1942a), which was designed for use with the macro Evelyn photoelectric colorimeter, could be adapted to the smaller quantities sufficient for use with microcuvettes. Even with the macro apparatus, 10 μl. serum is adequate for analysis. The principle of the Sendroy method is conversion of the chloride in acid solution to its silver salt by shaking with solid silver iodate; the iodate liberated by the chloride is made to act on potassium iodide, and the yellow color of the iodine set free is measured using filter No. 420 with the Evelyn instrument. For other methods see pages 200 and 281.

Sendroy Method for Chloride

SPECIAL REAGENTS

Approximately 0.085 M Phosphoric Acid. Test to make sure soln. is halide free.

Caprylic Alcohol.

Silver Iodate Powder, C. P. Test for presence of potassium iodate according to Sendroy (1939a): (*1*) Solubility measurement—Iodate analysis of a saturated soln. of silver iodate should give a value not exceeding 0.21 m*M*/l. (*2*) Analysis of a standard chloride soln.—Analyze a known 100 m*M* chloride soln. diluted twenty times with 0.085 *M* phosphoric acid.

5% Potassium Iodide. Test with starch to be sure no trace of free iodine is present.

PROCEDURE

1. Dilute the sample chloride soln. in 0.085 *M* phosphoric acid, or in tungstic acid soln. if protein is present, at between *p*H 2.0 and 3.0 to a final concentration of between 3 and 12 m*M*/l.

2. Add solid silver iodate (10 mg./ml.) to duplicate portions in 15 ml. centrifuge tubes, and shake vigorously for 2 min.; then either filter through halide-free paper or centrifuge for 1 min. at over 3000 R.P.M. The soluble iodate is now equivalent to the chloride in the sample.

3. Sendroy (1942a) recommended that the system given in Table I, B, of the paper by Sendroy and Alving (1942) be followed for the measurement of chloride in serum and blood. In this schedule 0.5–13 ml. iodate soln. containing 0.8 m*M* is added to 2.4 ml. 0.085 *M* phosphoric acid and 1.2 ml. 5% potassium iodide; the vol. is made up to 100 ml. with water.

4. Promptly after the color has been developed, transfer the tubes to a 25° water bath for 3–5 min. Wipe the tubes clean and dry; set the Evelyn instrument with filter No. 420 to read 100 with a blank soln., and then read the unknowns. The blank soln. may be prepared by omitting the iodate from the reaction mixture.

5. A calibration curve has been given by Sendroy and Alving (1942), but it is usually well to obtain one's own curve with the particular reagents and instrument used. The calibration may be made with standard potassium iodate solns.

PHOSPHATE AND PHOSPHATASE

Siwe (1935) has described the use of the Pulfrich step photometer with filter No. 72 (red) for the colorimetric measurement of inorganic phosphorus in small amounts of blood by conversion to phosphomolybdic acid and reduction by aminonaphtholsulfonic acid. The same reaction was employed by Weil and Russell (1940) in their phosphatase method (page 209). At about the same time Lundsteen and Vermehren (1936) developed a micro procedure for the determination of inorganic phosphate and alkaline phosphatase in blood plasma based on Müller's (1935) amidol reduction of phosphomolydic acid. The Pulfrich instrument with filter No. 72 was also used in this case, and for most measurements the 10 mm. cells were employed, but the 20 mm. cells were required for weaker colors. 50 μl. of blood are needed for a duplicate determination, and since the procedure might be adapted to tissue extracts as well, it will be described.

Conditions for the determination of inorganic phosphate in the presence of labile phosphate esters, such as phosphocreatine, acetyl phosphate, and ribose-1-phosphate, were established by Lowry and Lopez (1946). As these authors pointed out, the usual procedures for measurement of inorganic phosphate in tissue extracts represent the sum of the inorganic phosphate and the phosphate of the labile esters hydrolyzed by the reagents employed in the determination. The procedure of Lowry and Lopez is based on the reduction of phosphomolybdate by ascorbic acid at *p*H 4.0.

Bessey, Lowry, and Brock (1946) utilized as the substrate *p*-nitrophenyl phosphate, which had been studied by King and Delory (1939), and applied to phosphatase determinations by Ohmori (1937) and Fujita (1939). Bessey *et al.* were able to determine the phosphatase in as little as 5 μl. serum using 0.5 ml. of solution for the colorimetry. The advantage of this substrate is that it is colorless and yields the yellow salt of *p*-nitrophenol when the phosphate group is split off. Thus the color develops in proportion to the degree of the hydrolysis and no additional reagents are required for the color development. This advantage is also to be found in the use of phenolphthalein phosphate, which was employed by Huggins and Talalay (1945). However, alkaline phosphatase splits the *p*-nitrophenyl phosphate 25–30 times faster than the phenolphthalein com-

pound, 15% faster than phenyl phosphate, and 2–3 times more rapidly than glycerophosphate, according to Bessey, Lowry, and Brock. Either acid or alkaline phosphatase may be determined with the substrate; it is only necessary to carry out the colorimetry in alkaline solution, since the free nitrophenol, which would exist in acid solution, is colorless. For other methods see pages 124, 208, 209, and 280.

Lundsteen and Vermehren Method for Inorganic Phosphate and Phosphatase

SPECIAL REAGENTS

Substrate. Combine 8 ml. 1 *N* ammonium hydroxide, 12 ml. 1 *N* ammonium chloride, 1 g. disodium-β-glycerophosphate, 2 ml. 1 *M* magnesium chloride, and make up to 100 ml. with water.

10% Trichloroacetic Acid.

Acid-Molybdate Solution. Combine 100 ml., 7.5% ammonium molybdate, 45 ml. 10 *N* sulfuric acid, and 105 ml. water.

Amidol Solution. Dissolve 15 g. sodium sulfite and 1.5 g. Amidol (*Agfa*) in 100 ml. water. Store in the dark and cold, and dilute five times before use. After about 2 weeks it turns red and can no longer be used.

PROCEDURE

1. If blood is used, pipette 50 μl. into 1 ml. of 0.9% sodium chloride soln. and centrifuge out the cells.

2. To 200 μl. of the supernatant fluid or a tissue extract add 200 μl. substrate soln. and place in thermostat for the digestion period (for plasma 24 hr. at 37°).

3. To another tube containing the same ingredients add 300 μl. 10% trichloroacetic acid for a control experiment.

4. Stop the reaction by adding 300 μl. 10% trichloroacetic acid, and centrifuge out the precipitate from both the enzyme and control tubes.

5. To 400 μl. of the supernatant in each case add 100 μl. acid-molybdate reagent and 100 μl. Amidol soln.

6. Measure the color intensity after the soln. has stood for 15 min., and obtain the quantity of free phosphate from a previously determined calibration curve constructed from measurements with known amounts of phosphate.

Lowry and Lopez Method for Inorganic Phosphate in Presence of Labile Phosphate Esters

SPECIAL REAGENTS

Protein Precipitant. 5% trichloroacetic acid, or 3% perchloric acid, or (with very labile esters) saturated ammonium sulfate which is 0.1 *N* with respect to acetic acid and 0.025 *N* with respect to sodium acetate (*p*H 4).

0.1 N Sodium Acetate.

Acetate Buffer (*p*H 4). 0.1 *N* to acetic acid and 0.025 *N* to sodium acetate.

1% Ascorbic Acid.

1% Ammonium Molybdate in 0.05 N Sulfuric Acid.

0.05 mM Standard Phosphate Solution.

PROCEDURE

1. Deproteinize the sample with ice-cold protein precipitant. If either of the acid precipitants is used, bring the extract rapidly to *p*H 4.0 to 4.2 by adding 4 vol. of 0.1 *N* sodium acetate. (Most labile esters are fairly stable at this *p*H.)

2. Dilute the extract with the acetate buffer until the inorganic phosphorus concentration is 0.015 to 0.1 m*M* (0.05 to 0.3 milligram per cent). Dilute ammonium sulfate extracts at least five times.

3. Add 0.1 vol. 1% ascorbic acid and 0.1 vol. 1% acid-molybdate soln. to each vol. of extract. If used within 15 min. of their mixing, the ascorbic acid and molybdate may be combined.

4. Carry out the colorimetric reading at 5 and again at 10 min. after the addition of molybdate using light between 650 and 950 mμ (maximum absorption at 860 mμ).

5. Take simultaneous readings of the standard soln. and blank, which should be prepared parallel with the unknown. Should a difference be observed in the readings of the unknown at 5 and 10 min. compared to the standard, extrapolate the values to zero time.

NOTE: Lowry and Lopez have found the reaction to be delayed in the presence of certain tissue extracts. In these cases standardization must be obtained by adding a known quantity of inorganic phosphate to an aliquot and using the difference in the readings effected by the added phosphate for the standardization. Dilution overcomes the inhibitory effect to some degree. Thus, brain and muscle extracts should be diluted to a volume 150–200 times that of the tissue, and, in the case of liver 300–500 times.

Furthermore, acceleration in color development may be accomplished by increasing the molybdate to 1.5% in 0.05 *N* acid, and the ascorbic acid concentration to values that do not exceed a final concentration of 0.2%.

Bessey, Lowry, and Brock Method for Phosphatase

SPECIAL REAGENTS

Buffer-Substrate Solution. (*p*H 10.3 to 10.4). Prepare soln. *A* by dissolving 7.50 g. (0.1 mole) glycine and 95 mg. (0.001 mole) magnesium chloride in 700 to 800 ml. water; then add 85 ml. 1 *N* sodium hydroxide and dilute to 1 l.

Prepare soln. *B*, which is 0.4% disodium *p*-nitrophenyl phosphate in 0.001 *N* hydrochloric acid. (The authors reported that the *Eastman Kodak Co.* product contained about 50% inert material; hence twice the quantity of this preparation should be used. Purification may be carried out by recrystallization from hot 87% alcohol.) Adjust the *p*H of soln. *B* to 6.5 to 8.0 with acid or base, if necessary. Test for free nitrophenol by diluting 1 ml. with 10 ml. 0.02 *N* sodium hydroxide and measuring the absorption at 415 mμ. If the extinction is greater than 0.08 (*i.e.*, transmission less than 83% for 1 cm. liquid, or 70% for 2 cm.) remove the free phenol by extracting two to three times with equal vol. water-saturated butyl alcohol followed by three extractions with water-saturated ether. (All butyl alcohol must be removed since it inhibits phosphatase activity.) Aerate off the traces of ether and store in the cold.

Mix equal vol. of solns. *A* and *B*; adjust the *p*H to 10.3 to 10.4 if necessary with strong sodium hydroxide or hydrochloric acid, and store in the cold, or better, in the frozen state. When 2 ml. diluted with 10 ml. 0.02 *N* sodium hydroxide has an extinction greater than 0.1 for 1 cm., either discard or extract it with butyl alcohol, as above, and readjust the *p*H.

Standard Solutions. Prepare solns. containing 1, 2, 4, and 6 m*M* *p*-nitrophenol (molecular weight 139.1) per liter.

PROCEDURE

1. Place 5 μl. serum in the bottom of a 6 × 50 mm. serological tube, immerse in ice water, and rapidly add 50 μl. of the ice-cold buffer-substrate soln. with a constriction pipette. Mix by tapping with the finger.

2. Digest at 38° for 30 min., and then place in ice water and add 0.5 ml. 0.02 N sodium hydroxide with sufficient force to mix the solns.

3. Transfer to a cuvette and measure the color intensity using light at 400–420 mμ.

4. Add 2–4 μl. conc. hydrochloric acid and take a second colorimetric reading. The difference in the optical densities gives the corrected density of the unknown.

5. Run standards and blanks by treating 5 μl. vol. of the standards and distilled water in the same manner as the serum. Construct a standard curve from the corrected optical densities. If the same pipettes are used for both the standards and unknowns, the exact pipette vol. need not be known.

Bessey *et al.* employ a "millimole unit" which is defined as the phosphatase activity which will liberate 1 mM nitrophenol/liter/hr. 1 millimole unit is approximately equal to 1.8 Bodansky units. For sera weaker in phosphatase, such as those from adults, the vol. serum and reagent may be doubled without increasing the vol. alkali; this will yield a color of nearly double the intensity.

NITROGEN AND AMMONIA

General

As in the more macro methods, the determination of nitrogen in histochemical investigations involves conversion of the total nitrogen to ammonium sulfate by digestion. The digest may be Nesslerized directly, or it may be alkalized and the liberated ammonia absorbed in acid and measured either colorimetrically or titrimetrically. However, the determination of the very small quantities involved requires unique treatment. Since the preliminary procedures are common to both the colorimetric and titrimetric methods, it will perhaps contribute to greater integration and clarity if the chief developments in both forms of analysis are considered together in chronological order at this point.

At about the same time, Linderstrøm-Lang and Holter (1933b) and Conway and Byrne (1933) reported methods for the microestimation of ammonia which depended on the transfer, by diffusion, of the ammonia from an alkalized solution to one of standard acid followed by titration. Linderstrøm-Lang and Holter employed a

tube having a total capacity of about 250 μl. for the diffusion process. The sample was placed in the bottom of the tube and the ammonia from it was allowed to diffuse into a drop of standard acid placed in the upper part of the tube to form a seal across the lumen (Fig. 46). Conway and Byrne used a special diffusion cell (Fig. 41) requiring considerably more of the liquids; hence it was not suitable for the accurate measurement of much less than 14 μg. ammonia nitrogen. The Linderstrøm-Lang and Holter method had a precision of 0.005 μg. nitrogen and it could be used for the determination of up to about 28 μg. The following year Gibbs and Kirk (1934) employed a modified Conway-Byrne procedure, which they used for the estimation of from 1.5–8.3 μg. ammonia nitrogen. Conway (1935b) subsequently described a refinement of the diffusion cell method which had an ultimate standard deviation of 0.02 μg. ammonia nitrogen.

Levy (1936) developed a technique for the determination of total nitrogen based on the direct Nesslerization of the acid-digested sample. The complete treatment was carried out in the same vessel and the final solution was transferred for colorimetry to a microcuvette having a capacity of 0.2 ml. Levy's method was adapted for quantities of nitrogen in the range 0.5–6.0 μg. and the average deviation observed was 0.03 μg.

A titrimetric method for total nitrogen employing features of both the Linderstrøm-Lang and Holter and the Conway techniques was published by Needham and Boell (1939). These investigators used a single vessel with a special cap for all the operations, *i.e.*, digestion, diffusion, and titration. The method was adapted to 1–20 μg. of nitrogen and the standard deviation in control experiments was 0.3 μg. In order to refine the earlier colorimetric method of Borsook (1935), Borsook and Dubnoff (1939) also borrowed features of the Linderstrøm-Lang and Holter technique as well as the diffusion cell of Conway and Byrne to develop a method for total nitrogen, ammonia, and other nitrogenous compounds. The procedure of Borsook and Dubnoff for 5–10 μg. total nitrogen involves acid digestion, transfer of an aliquot to a diffusion cell, and finally electrometric titration of the excess acid. The standard deviation was around 0.05 μg.

Levy and Palmer (1940) adapted the hypobromite method for ammonia to the iodometric estimation of nitrogen without diffusion.

The principle of this method, which was originally proposed by Rappaport (1935), is iodometric measurement of the excess hypobromite remaining after reaction with ammonia according to the equation: $3\ NaOBr + 2\ NH_3 \rightarrow N_2 + 3\ H_2O + 3\ NaBr$. The procedure of Levy and Palmer can be used for total nitrogen in the range 500–5 μg. or, if the microvolumetric techniques of Linderstrøm-Lang and Holter are used, down to 0.5 μg. After digestion in small test tubes the material is diluted with water, made alkaline with a neutralizing reagent, treated with an excess of hypobromite, and this excess is measured by the addition of potassium iodide and titration with thiosulfate. The entire treatment can be carried out in one small tube.

As an improvement on the diffusion procedure of Bentley and Kirk (1936), Tompkins and Kirk (1942) described a specially constructed unit for both digestion and diffusion designed for the measurement of samples containing 0.5–20 μg. nitrogen. A probable error of not more than about 1% was claimed by the authors, but this was challenged by Hawes and Skavinski (1942), who stated that the diffusion time employed by Tompkins and Kirk is sufficient to transfer only about 90% of the ammonia in samples containing less than 10μg. nitrogen.

Hawes and Skavinski (1942) described another modification of the diffusion cell technique. They employed a test tube for both digestion and diffusion. A small helix of platinum wire, which was sealed to a glass tube held in a rubber stopper, was used to hold a drop of the ammonia-absorbing solution. A 1 *M* primary sodium phosphate solution, rather than the commonly used saturated boric acid solution, was employed to absorb the ammonia because of the greater absorption capacity of the former. The volume of phosphate solution need not be measured; it is sufficient merely to dip the helix into the stock solution. After the absorption is complete, the helix is immersed in water and the solution is titrated with standard acid to an end point between *p*H 4.3 and 4.7. Electrometric titration is advantageous but an indicator may be used; in the latter event a bromocresol green-methyl red mixture is superior to the methylene blue-methyl red combination. As used by Hawes and Skavinski, the method enables the determination of from 10 to 100 μg. nitrogen.

Russell (1944), by utilization of the Conway-Byrne diffusion cell for the collection of ammonia, refined the phenol-hypochlorite

method for the colorimetric determination of ammonia, which had been used earlier by Van Slyke and Hiller (1933) and Borsook (1935). By the reaction of ammonia with phenol and hypochlorite in alkaline solution an intense blue product is formed, which is believed to be indophenol or a closely related compound. The method has been applied to 1.5 ml. ammonia solution containing 0.5–6.0 μg. nitrogen. Larger or smaller volumes may be used to extend the range of the method.

Boell (1945) employed a method for the estimation of 1–50 μg. total nitrogen which utilized a digestion similar to that of Levy (1936), transfer of an aliquot of the digest to a diffusion unit made up of ordinary laboratory glassware rather than the special cells of Conway and Byrne, and titration with a simplified microburette. The standard deviation was about 0.1 μg. nitrogen.

The method of Linderstrøm-Lang and Holter (1933b) for ammonia has been adapted to the determination of total nitrogen and subjected to exhaustive critical trial and investigation in the Carlsberg Laboratory. Changes have been introduced as dictated by experience and a description of the procedure used in 1939 was given in a publication of Bottelier, Holter, and Linderstrøm-Lang (1943). A final summary of the method after further improvements, with a full treatment of each step, was the subject of a paper by Brüel, Holter, Linderstrøm-Lang, and Rozīts (1946). In principle, the final method consists merely of the digestion of the sample, transfer of the digest to the bottom of a paraffin-coated tube, and titrimetric measurement of the ammonia in a manner similar to that originally employed by Linderstrøm-Lang and Holter but with improvements in certain of the details.

The preceding survey indicates that various procedures may be used for every step in the nitrogen determination. The choice of method may depend to some degree on the prejudices and preferences of the experimenter, but the method of Brüel *et al.* (1946) is recommended as the most foolproof and reliable.

Titrimetric Methods. The Levy and Palmer (1940) method has the great advantage of employing a simple small tube for all of the chemical steps, and the diffusion process is avoided altogether. Certainly the digestion of the sample can be performed in a small tube regardless of the ensuing procedure. If an ammonia diffusion is to be employed, it is simpler to use small tubes for it rather than

the specially constructed vessels of Conway and Byrne (1933), Needham and Boell (1939), or Tompkins and Kirk (1942). In this regard, advantages might be claimed for the method of Brüel *et al.* (1946), in which both diffusion and titration are carried out in the same simple tube, or the Hawes and Skavinski (1942) method, in which the digestion and diffusion are conducted in the same tube. However, the former requires transfer of digest and the latter transfer of acid. The method of Brüel *et al.* (1946) is the most precise of all (0.005 μg. nitrogen), and the one most thoroughly tested for its reliability. This method is described on page 283.

Colorimetric Methods. Levy's (1936) method for the direct Nesslerization of the digest has the advantage that all of the chemical operations may be carried out in the same vessel and the diffusion process is eliminated. On the other hand, the phenol-hypochlorite reaction used by Russell (1944) is highly sensitive, and ammonia, in the quantities that can be determined by Levy's method, can be analyzed with ordinary macro equipment. However, diffusion of the ammonia from the digest is essential for a proper phenolhypochlorite reaction.

Digestion of Sample for Determination of Total Nitrogen

The digestion can be conveniently performed in small tubes of resistant glass. In the earlier methods after the sample had been introduced, a small Hengar granule (*Hengar Co.*) was sometimes added to prevent bumping, the digestion mixture was introduced, and the tubes were placed in a drying oven at 120–130° for a few hours, or at 105–110° overnight, to drive off most of the water. The digestion was continued by heating the tubes in a sand bath, or in a copper or aluminum block with holes drilled so that the tubes could be inserted to a depth not exceeding one third of the tube length.

Bruel, Holter, Linderstrøm-Lang and Rozīts Procedure for Digestion of the sample. In the procedure of Brüel *et al.* (1946), which should be given preference, the digestion tubes are 4 cm. long, 1.8 mm. inner diameter, and 2.4 mm. outer diameter. The tubes are cleaned by boiling in sulfuric acid-chromic acid mixture, rinsed thoroughly with distilled water run through each tube by means of a thin capillary extending to the bottom, and the water is

removed by suction through a thin capillary. The tubes are dried *in vacuo* at 100° for 10 min. The rims of the tubes are never touched with the fingers after removal from the cleaning solution and the clean tubes are stored in a desiccator or in petri dishes in a room where no ammonia is allowed and smoking is prohibited. To the sample in a tube about 4 μl. of the following mixture is added with a constriction pipette, properly centered so that the solution delivered to the bottom of the tube is not drawn up between the pipette and the inner wall:

1 g. copper sulfate ($CuSO_4 \cdot 5H_2O$)
10 g. potassium sulfate
0.2 g. sucrose
5 ml. conc. sulfuric acid
Dilute to 100 ml.

(The sucrose is added to make sure that the reduction of the nitrogenous impurities in the reagents also takes place in the blanks.) The constriction pipette is emptied under constant pressure, which, for the greatest accuracy, is so adjusted that the pipette automatically empties when the tip touches a wall or liquid surface. Before use the pipette is rinsed inside and out with distilled water. After the digestion solution is added to the sample the tubes are placed in a vacuum desiccator over phosphorus pentoxide and the water is allowed to evaporate at room temperature. To prevent creeping the bulk of the water is removed at 150 mm. mercury (about 24 hr.); then the drying is continued at 0.1 mm. mercury (another 24 hr.).

By means of a mouth-operated horizontal pipette with a vertical delivery tip, 1 μl. conc. sulfuric acid containing 10 mg. selenium/ml. is added to the charred sample in the digestion tube. (About each 10 mm. of the graduated length of the pipette corresponds to 1 μl.; a strip of graph paper may be used for the graduation. The selenium-acid solution is prepared by boiling until clear.) Proper centering of the delivery tip is essential to prevent the liquid from being drawn up between the tip and the wall of the tube. Should the liquid be drawn up as far as the rim where contamination may occur, the experiment should be discarded. It is also essential to rinse the pipette both inside and outside each day before use.

The digestion is carried out in small flasks (Fig. 78), which may be made by forming a constriction in the middle of insulin ampules. 1 ml. conc. sulfuric acid containing 0.4 g. potassium sulfate is placed

in the bottom of the flask, and before being used for the first time the solution is boiled in the flask to drive off water and gases. A small glass ball (Fig. 79) is used to close the flask when not in use. The flasks are heated conveniently in a copper block supplied with an electric coil. Holes in the block permit the flasks to be inserted to a depth of about 14 mm.; the temperature is kept constant at 295°. At this temperature the acid is clear; it fumes but does not boil.

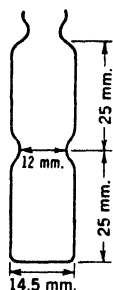



Fig. 78. Arrangement for digestion.

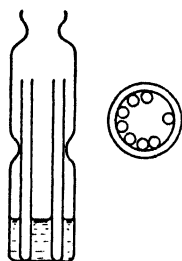



Fig. 79. Glass stopper for digestion flask.

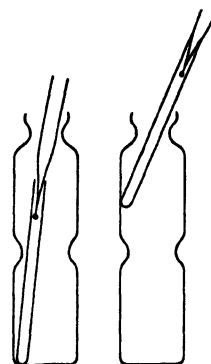



Fig. 80. Removal of tubes from digestion flask.

From Brüel et al. (1946)

To insert the digestion tubes the flask is removed from the block, allowed to cool for 30 sec., and then the tubes are inserted by means of forceps and arranged in a row around the circumference of the flask (Fig. 78). After replacement of the flasks in the block the digestion is watched for a few minutes to see whether liquid seals form across the lumen of the tubes and rise upward. If they rise higher than the middle of a tube the flask is removed from the block for a minute or two and the seals collapse. After the initial stage the digestion is allowed to proceed unattended for 5–6 hr. The tubes are removed from the slightly cooled flasks by means of a conical glass rod as indicated in Figure 80. They are rinsed on the outside with distilled water, dried with a clean towel, and stored in a desiccator over phosphorus pentoxide until ready for the determination of ammonia.

Digestion Mixtures Used by Other Workers. 1 ml. conc. sulfuric acid, 0.625 g. potassium sulfate, and 0.075 g. selenium; dilute about 5 times.—Levy (1936).

1% selenium dioxide and 1% copper sulfate ($CuSO_4.5H_2O$) in concentrated sulfuric acid and water (1:1); after digest is colorless, cool, add 1 drop saturated potassium persulfate, and continue digestion for 15 min. after the solution becomes water clear.—Borsook and Dubnoff (1939).

3 g. copper sulfate, 1 g. potassium sulfate, and 0.1 g. selenium dioxide to 300 ml. conc. sulfuric acid.—Needham and Boell (1939).

Conc. sulfuric acid and water (1:1); when fuming starts, add 1 drop of 30% hydrogen peroxide. After digest is clear, cool, add a couple of drops of saturated potassium persulfate, and continue heating; destroy peroxides by adding water, after cooling, add another Hengar granule, and place in an oven at about 120° for 30 min.—Levy and Palmer (1940).

Conc. sulfuric acid and water (1:1) saturated with potassium sulfate and made 0.1% with respect to copper selenite. The latter is prepared by mixing a strong copper sulfate soln. with one of sodium selenite and collecting the precipitate.—Tompkins and Kirk (1942).

18 *N* sulfuric acid containing 0.1% selenium dioxide and 0.1% copper sulfate; use 30% hydrogen peroxide if needed.—Hawes and Skavinski (1942).

1 g. copper sulfate, 1 g. potassium sulfate, 1 g. selenium dioxide, and 100 ml. 50% sulfuric acid; add saturated potassium persulfate to the cooled mixture if required.—Boell (1945).

Levy Nesslerization Method for Nitrogen

SPECIAL REAGENTS

Digestion Mixture. See above.

Nessler Reagent (Folin and Wu). Add 15 g. potassium iodide and 11 g. iodine to 10 ml. distilled water and introduce an excess of mercury (14–15 g.). Shake well for 7–15 min. or until the dissolved iodine has nearly all disappeared. Cool in running water when the solution begins to become pale and continue shaking until the greenish color of the double iodide ($HgI_2.2KI$) appears. Decant the solution and wash out vessel with distilled water. Dilute the solution and washings to 200 ml. Add 75 ml. of this solution and

75 ml. distilled water to 350 ml. 10% sodium hydroxide (within 1% of 2.5 *N*) to make the final Nessler reagent.

PROCEDURE

1. Place a microtome section of tissue in 7 μl. water in the bottom of a digestion tube (Fig. 39, page 167). This tube has a total capacity of about 2.5 ml. Add 50 μl. of the Levy digestion mixture and drive off most of the water at 130° (about 1.5 hr. required). Digest until water-white (Levy uses a small mobile gas flame).

2. After the tube has cooled, hold almost horizontally and pipette 700 μl. distilled water directly into the digest, delivering the water with as much force as possible. If complete solution is not attained at once, it will occur on standing.

3. Add the Nessler solution in a standardized manner (Levy, 1936): First, "A piece of glass tubing is drawn out to a long capillary and clamped vertically above a platform which may be raised and lowered by a rack and pinion. It is connected to a source of air supply at 20 cm. water pressure. The capillary is of such diameter that a rapid stream of air bubbles passes through when immersed in water." Then, the reaction tube is placed in a holder and set on the platform under the capillary tube with air flowing through it. Fill a 300 μl. constriction pipette with Nessler reagent and place one end of a rubber tube connected to the pipette in the mouth, and hold the pipette in the right hand. "The pinion of the platform is now turned with the left hand to raise the tube about the bubbler so that a stream of air bubbles passes from bottom to top of the solution. Just as soon as the bubbler reaches the bottom the Nessler reagent is blown in. The platform is then lowered. The entire process takes five seconds." Tilt the tube back and forth to collect any solution in the bulb.

4. Make the colorimetric measurement from 10–90 min. after Nesslerizing. Prepare a blank by following the preceding steps, but omitting the sample. (Levy used a Pulfrich photometer with filter S43. The microcuvettes which hold 0.2 ml. were employed.)

Russell Phenol-Hypochlorite Method for Ammonia

SPECIAL REAGENTS

Alkaline Phenol Reagent. Add some water to 25 g. crystalline phenol and pour in with stirring 54 ml. 5.0 *N* sodium hydroxide.

Make up to 100 ml. and store in a brown bottle in a refrigerator.

Hypochlorite Solution. Dissolve as much as possible of 25 g. of ground and sifted calcium hypochlorite in 300 ml. hot water. With stirring, add 135 ml. 20% potassium carbonate soln. which had been boiled to drive out ammonia. Heat briefly to about 90°, cool, and dilute to 500 ml. Filter a little of the soln. and test for calcium ion by adding some of the potassium carbonate soln. and heating in boiling water a few min. In the absence of calcium ion the solution remains crystal clear. If the test is positive, add more carbonate until a negative test is obtained. Filter the final soln. and store in small brown bottles in a refrigerator. This soln. should be water clear and contain 1.30–1.40% free chlorine. Test for free chlorine by adding 10 ml. water, 2 ml. 5% potassium iodide, and 1 ml. glacial acetic acid to 2.00 ml. of the hypochlorite solution. Titrate with 0.100 *N* thiosulfate; 7.5–8.0 ml. should be required. Occasionally retest the solution. The soln. may also be prepared from Clorox (page 58),

0.003 M Manganous Chloride or Sulfate.

PROCEDURE

1. Place 1.5 ml. sample in neutral or acid solution not stronger than 0.01–0.02 *N* (containing 0.5–6.0 μg. ammonia nitrogen) in a colorimeter tube, cooled by an ice bath. Add 1 drop manganous salt soln., 1 ml. cold alkaline phenol reagent, and 0.5 ml. cold hypochlorite solution. Mix by gentle rotation and place at once in a boiling water bath for about 5 min.
2. Cool, dilute to a convenient volume such as 6 or 10 ml., and measure the color intensity with light of about 625 mμ.

URIC ACID, CREATINE, CREATININE, AND ALLANTOIN

Borsook (1935) reported colorimetric methods for uric acid, creatine, creatinine, and allantoin which were modifications of procedures already in use, but which are in the category of micro methods, even though no special micro equipment is required. The colorimetric measurements were carried out spectrophotometrically in cuvettes taking 3.0–3.5 ml. With the present availability of microcuvettes, these methods could be adapted to the analysis of much smaller quantities of the substances by substituting smaller test tubes for the 125 × 9 mm. (inside dimensions) tubes used.

The uric acid method is based on precipitation of the substance with zinc, solution of the precipitate in dilute acid and water, addition of cyanide followed by Benedict's arsenophosphotungstic acid reagent, and development of the color by a procedure which yields a clear solution. The method was designed for the analysis of 1 ml. of sample having less than 1 milligram per cent uric acid, *i.e.*, less then 10 μg. For another method see page 213.

The creatinine method involves absorption of the compound on Lloyd reagent, removal of impurities, and liberation of the creatinine by the same alkaline picrate in which the color is developed. Creatine is determined by the increase in creatinine after conversion to the latter. With the 3 ml. cuvettes which Borsook used, the smallest quantity of creatinine which could be measured was 1 μg. The absolute error with all concentrations was ±0.1 μg. For another method see page 211.

The allantoin method depends on the enzymatic conversion of allantoin to allantoic acid (in the presence of cyanide to prevent the formation of allantoin from uric acid), acid hydrolysis of the allantoic acid to urea and glyoxylic acid, and colorimetric measurement of the latter. With 2 ml. of sample, the least that can be measured is 0.05 milligram per cent (1 μg.) with an error of ±5%.

Sure and Wilder (1941) employed the micro attachment on the Evelyn photoelectric colorimeter for the measurement of creatine and creatinine. Gold-plated plungers had to be used because the ordinary cadmium-coated plungers were corroded by the saturated picric acid. The error in the conversion of creatine to creatinine by the procedure employed varied from —0.79 to +2.66% in test experiments. 1 ml. blood filtrate was used by the authors for each analysis.

Borsook Method for Uric Acid

SPECIAL REAGENTS

2.5% Zinc Chloride.

10% Sodium Carbonate.

N/14 Hydrochloric Acid.

5% Sodium Cyanide (containing 2 ml. conc. ammonium hydroxide per liter. Prepare fresh every 6–7 weeks.

Benedict's Arsenophosphotungstate Reagent. Dissolve 100 g. pure

sodium tungstate in 600 ml. water. Add 50 g. pure arsenic pentoxide followed by 25 ml. 85% phosphoric acid and 20 ml. conc. hydrochloric acid. Boil for 20 min., cool, and dilute to 1 liter.

Folin's Stock Uric Acid Standard Soln. Place 1 g. uric acid, weighed to 1 mg., in a 1 l. volumetric flask. Add 150 ml. water to 0.6 g. lithium carbonate in a 250 ml. flask and shake until dissolved. Filter, and heat the filtrate to 60°. Warm the flask containing the uric acid in running hot water and pour the warm lithium carbonate soln. into the volumetric flask containing the uric acid; wash down crystals adhering to the neck. Shake until the uric acid has dissolved (about 5 min.), cool under the tap, add 20 ml. 40% formaldehyde, and half fill the flask with water. Add a few drops of methyl orange and then pipette in slowly, with shaking, 25 ml. 1 *N* sulfuric acid. Dilute the pink soln. to 1 l. Store the reagent in well-stoppered bottles and keep in the dark. Diluted standards made from this soln. will keep for several days, but do not use them sooner than 1 hr. after they are made.

PROCEDURE

1. Pipette 1–2 ml. of sample, 1 ml. water, and 0.05 ml. 2.5% zinc chloride into a Pyrex test tube (125 × 9 mm. inside) provided with a ground-glass stopper. Mix well by inversion several times.
2. Add 0.4 ml. 10% sodium carbonate and again mix by inversion.
3. Centrifuge the test tube, pour off the supernatant, and take up the last drop from the lip of the tube with filter paper.
4. Add 0.5 ml. *N*/14 hydrochloric acid, 1.5 ml. water, and 1 ml. cyanide soln.
5. Stopper the tube and shake well to dissolve all precipitate and leave a clear colorless soln.
6. Add 0.2 ml. arsenophosphotungstate soln. and again mix well by inversion.
7. Place the stoppered tube in a 37° water bath for 40 min., and then in an ice water bath for 15 min.
8. Centrifuge, transfer some of the supernatant to an absorption cell, and determine the color spectrophotometrically at 610 mμ.
9. In a parallel manner, treat four standard solns. covering the range 0 to 1.0 milligram per cent uric acid in order to obtain a calibration.

Borsook Method for Creatine and Creatinine

SPECIAL REAGENTS

0.1 N Hydrochloric Acid.

0.01 N Hydrochloric Acid.

Lloyd Reagent (Eli Lilly and Co.).

Alkaline Picrate Solution. Combine 10 parts saturated picric acid and 1 part 10% sodium hydroxide.

Creatinine-Zinc Chloride Standard. Dissolve 1.61 g. creatinine-zinc chloride (Benedict, 1914) in *N*/14 hydrochloric acid and make up to 1 l. This soln. has 1 mg. creatinine/ml. Make up fresh at least once a month.

PROCEDURE

1. Convert creatine to creatinine: Place 1–5 ml. of sample in a Pyrex test tube (125 × 9 mm. inside) provided with a ground-glass stopper, add one fourth the vol. of 0.1 *N* hydrochloric acid and mix by inversion. Insert a piece of thread into the neck of the tube and stopper. Then autoclave for 20 min. at 30 lb. (130°). Omit this step for preformed creatinine.
2. After cooling, add 30–40 mg. Lloyd reagent and, with thread removed, stopper tightly and shake continuously for 10 min.
3. Centrifuge, pour off supernatant, and remove the last drop from the lip of the tube with filter paper.
4. Resuspend the precipitate in 1 ml. 0.01 *N* hydrochloric acid and use another 1 ml. portion of the acid to wash down the stopper and the sides of the tube.
5. Again centrifuge and discard the supernatant, removing the final drop from the lip with filter paper.
6. Add 3 ml. sodium picrate soln. to remove the creatine from the Lloyd reagent, and shake gently for 10 min. to develop the color fully.
7. Centrifuge, and measure the color of the liquid spectrophotometrically at 525 mμ. A linear relationship exists between the absorption and the creatinine concentration from 0 to 2.0 milligram per cent.
8. Adsorb standards on Lloyd reagent and treat as above from step 2 on.

Sure and Wilder Method for Creatine and Creatinine

SPECIAL REAGENTS

Saturated Picric Acid.
10% Sodium Hydroxide (carbonate free).

PROCEDURE

1. Pipette 1 ml. of sample (blood filtrate) into a 15 ml. centrifuge tube and add 0.5 ml. picric acid soln.
2. To convert creatine to creatinine, cover the tube with lead foil and autoclave for 40 min. at 20 lb. pressure. Omit this step for preformed creatinine.
3. Cool, add 0.1 ml. of the sodium hydroxide, and allow to stand for 10 min.
4. Run a control on the reagents alone.
5. Using the Evelyn photoelectric colorimeter, set the control at 100 with filter 520-M, and then obtain readings of the unknown. Do not allow the plungers to remain in contact with the soln. for longer than 1–2 min. in order to avoid corrosion.
6. Obtain values from a previously established calibration curve.

Borsook Method for Allantoin

SPECIAL REAGENTS

Enzyme Powder. Use urease preparation (*Squibb*) from soy bean meal (Van Slyke and Cullen, 1914).
Ammonium Carbonate-Sodium Cyanide. Dissolve 1.153 g. ammonium bicarbonate, 0.891 g. ammonium carbonate, and 0.46 g. sodium cyanide in water and make up to 200 ml.
10% Trichloroacetic Acid.
2% Sodium Tungstate.
N/15 Sulfuric Acid.
0.5% Phenylhydrazine Hydrochloride in *N*/14 hydrochloric acid. Dissolve commercial phenylhydrazine hydrochloride in water and decolorize by boiling with activated charcoal. Filter the hot solution, and, after the filtrate is cooled in an ice-salt bath, precipitate the phenylhydrazine hydrochloride by addition of conc. hydrochloric acid, or by dry hydrochloric acid gas. Filter the precipitate off by suction; wash once quickly with very cold hydrochloric acid,

and place in a desiccator over calcium oxide in the dark. Make up the soln. just before use.

1.25% Potassium Ferricyanide. Prepare just before use.

Standard Solution. Prepare fresh each week a soln. of 1 mg. allantoin/ml.

PROCEDURE

1. Into a Pyrex test tube (125 × 9 mm. inside) provided with a ground-glass stopper place 10 mg. dry enzyme powder, 0.5 ml. carbonate-cyanide soln., 2 ml. of the sample to be analyzed, and a drop of chloroform.
2. Stopper the tube and let stand at 37° for 12 hr. An occasional shaking aids in the solution of the enzyme.
3. Transfer 2 ml. of the mixture into another test tube and add 0.2 ml. 10% trichloroacetic acid and 0.1 ml. 2% sodium tungstate. Mix after stoppering by inverting a few times. Then add 0.1 ml. *N*/15 sulfuric acid and again mix by inversion.
4. Place tube in a large beaker of water at room temperature and heat the water quickly to 90° for 5 min. Cool quickly in ice water for 2 min.
5. Add 0.3 ml. 0.5% phenylhydrazine soln., stopper and shake vigorously.
6. Set in a water bath at 60° for 5 min. and quickly cool in ice water.
7. Centrifuge; carefully float a drop of alcohol on the surface of the liquid, and again centrifuge to throw down floating particles.
8. Carefully pipette 2 ml. clear supernatant into another dry test tube and cool it in a dry ice-alcohol mixture contained in a beaker surrounded by an ice-salt bath maintained at —15 to —20°.
9. Cool some conc. hydrochloric acid in the Dry Ice-alcohol mixture, and, after the tube has been cooling for about 10 min., add 1.5 ml. of the cold hydrochloric acid to it.
10. Stopper, and continually invert in the air until the frozen material melts.
11. Just before the last of the frozen material disappears add 0.2 ml. potassium ferricyanide soln. and mix quickly by several inversions.
12. Set in an ice-salt bath for 5 min. and then place the tube in a beaker of water at room temperature.

13. After 10 min., promptly measure the color (it slowly fades and becomes turbid) spectrophotometrically at 535 mμ. From 0 to 1.5 milligram per cent allantoin there is a linear relationship between absorption and concentration.

14. Run the standard soln. in the same manner and at the same time as the unknown.

ASCORBIC ACID*

The method of Roe and Kuether (1943) for assay of ascorbic acid was adapted by Lowry, Lopez, and Bessey (1945) to determinations on amounts of blood serum down to 10 μl. Measurements in the range 0.3 to 1.4 milligram per cent have been made with a standard deviation, in single determinations, of the order of 0.03 milligram per cent. Ascorbic acid is converted to dehydroascorbic acid, the latter is treated with 2,4-dinitrophenylhydrazine, and the osazone formed is made to yield a colored dehydration product through the action of sulfuric acid. Pijoan and Gerjovich (1946) pointed out that, while this method is reliable for use on blood, its application to tissues must be made with caution in regard to oxidation products of ascorbic acid. This follows since the phenylhydrazine reaction is not specific for dehydroascorbic acid but can react with structures, such as diketogulonic acid, which bear no antiscorbutic properties. Before applying the procedures to tissues, it would be desirable, and perhaps necessary, to ascertain in advance whether interfering substances were present in the tissue. A titrimetric method, employing dichlorophenol indophenol, which measures ascorbic acid directly and not dehydroascorbic acid, is given on page 300.

Lowry, Lopez, and Bessey Method for Ascorbic Acid

SPECIAL REAGENTS

Osazone Reagent. Prepare a soln. of 2% dinitrophenylhydrazine and 0.25% thiourea in 9 *N* sulfuric acid; centrifuge or filter through sintered glass if a precipitate develops. Store in a refrigerator and discard after 1 month.

65% Sulfuric Acid. Add 70 ml. of the concentrated acid to 30 ml. water.

* See Bibliography Appendix. Refs. 33, 34, and 43.

1% Suspension of Norit in 5% Trichloroacetic Acid. First treat the Norit by placing 200 g. in a large flask, add 1 l. 10% hydrochloric acid, heat to boiling, filter with suction, transfer the cake of Norit to a large beaker and add 1 l. distilled water; stir well, filter, and repeat the whole procedure until the washings give a negative or very faint test for ferric ion. Dry overnight at 110–120°. Some grades of activated carbon may not require washing; this can be determined by running a blank test on trichoroacetic acid washings of the carbon. If these give no more color than the acid alone, the washing of the carbon is unnecessary. Suspend 5 g. of the iron-free Norit in 100 ml. 5% trichloroacetic acid. After the Norit has settled, decant the supernatant and restore the volume with 5% trichloroacetic acid. Repeat several times to eliminate some of the very fine floating carbon particles. It is necessary to prevent carbon from getting into the final sample since carbon contamination may result in low values. If difficulty from floating is encountered, add 1 vol. 2% gelatin to 10 vol. of the acid suspension just before use. Once a week or so, replace the supernatant by fresh acid to avoid the possibility of contamination with heavy metals that may slowly leach out of the Norit.

PROCEDURE

1. Place 10 μl. serum in the bottom of a serological tube (6 $\times$ 50 mm.); add 40 μl. of the acid-charcoal suspension, and mix by tapping the tube. In pipetting the charcoal suspension, first blow through the pipette to suspend the material and then fill and empty it rapidly to prevent the charcoal from settling out. Employ a constriction pipette with a tip and constriction two to three times wider than normal to avoid plugging.
2. Cap the tube with a piece of Parafilm or a stopper and centrifuge 10 min. at 3000 R.P.M.
3. Transfer 30 μl. of the supernatant to another serological tube; add 10 μl. of the osazone reagent, and mix by tapping.
4. Cap the tube and set aside at 38° for 3 hr.
5. Chill the tube in ice water and add 50 μl. ice-cold 65% sulfuric acid. Mix very well, and, after 30 min. at room temperature, measure the color intensity at 520 mμ using the 0.05 ml. cuvette with a Beckman spectrophotometer (page 216). If the vol. is increased the 0.2 ml. cuvette may be used.

6. Prepare a standard and blank by adding 4 ml. of the acid-charcoal suspension to 1 ml. aliquots of freshly prepared 1 milligram per cent ascorbic acid soln. and water, respectively. Centrifuge and treat 30 μl. aliquots in the same fashion as the unknowns. Take care to avoid floating charcoal, which is more of a problem in the absence of serum. Correct both standard and unknown for the blank and calculate the result; only the single standard is required since the color is directly proportional to the concentration of ascorbic acid.

NOTE: Serum may be stored safely for several days in a refrigerator or for several weeks at −20° after the acid has been added. The supernatant acid extract may be separated and stored safely in a refrigerator for at least several weeks, and presumably at −20° for an indefinite period. The tubes must be well sealed with rubber stoppers to prevent evaporation. When samples are stored in the frozen state it is preferable to separate the supernatant before freezing in order to avoid the troublesome tendency for the charcoal to float as a result of the subsequent necessity for stirring. The rapid loss of ascorbic acid in blood at ordinary temperatures makes it imperative to keep the material cool until it is acidified.

In the preceding method of Lowry *et al.* the danger of charring has been minimized by the use of 65% sulfuric acid instead of the 85% acid used in the original method of Roe and Keuther; however, it is still necessary to cool the reaction mixture. Bolomey and Kemmerer (1946) suggest the substitution of glacial acetic acid for the sulfuric acid in order to avoid the danger of charring without the necessity of cooling. The color intensity developed with the acetic acid is about half that obtained with the sulfuric acid, which may or may not be important.

GLYCOGEN

While colorimetric methods for glycogen have not been developed specifically for histochemical studies, the procedure of Boettiger (1946) can be adapted to the micro scale necessary.

In the method of Boettiger, the glycogen obtained by alcohol precipitation of an alkaline digest of the tissue is dissolved, heated with an acid solution of diphenylamine, and the color which is developed is measured (a filter No. 635 is used with the Evelyn photoelectric colorimeter). This method enables duplicate determinations on 5–10 μg. glycogen, and if reduced to the volumes required in micro-

cuvettes, will enable the corresponding refinement. The chief difficulty is the erratic behavior of the diphenylamine reagent, which necessitates a new calibration each time the determinations are carried out.

The method of van Wagtendonk *et al.* (1946) is based on the color development which occurs when Lugol solution is added to the glycogen isolated from the tissue. A Klett-Summerson photoelectric colorimeter was used with filter No. 54 and the measurements were made in the range 0.05–2 mg. glycogen in a total volume of 5 ml. Morris (1946) has pointed out that the color developed with iodine varies considerably with temperature, and therefore temperature control is required for accurate work. The concentration of iodine is also a factor that affects the color intensity, and the importance was stressed for standardization with glycogen obtained from the same source as the material to be analyzed. Morris is of the opinion that the precautions required to render the iodine method sufficiently accurate constitute a major disadvantage. Nevertheless, if temperature is controlled and care is taken to maintain a constant iodine concentration the method should yield reproducible results. If, in addition, the standard solution is prepared from glycogen native to the tissue to be analyzed, sufficient accuracy should be obtained.

For work on the histochemical level, the tissue may be digested with alkali and the glycogen precipitated with alcohol according to steps 1–7 in Heatley's procedure (page 299). The glycogen thus isolated can be treated in the manner used by Boettiger or van Wagtendonk *et al.* Some preliminary work will be required, no doubt, to obtain the proper color intensities for the apparatus employed.

Boettiger Method for Glycogen

SPECIAL REAGENTS

Diphenylamine Reagent. The purest diphenylamine must be used. Oxidized crystals are brownish and impart a blue color to the reagent. The compound can be purified by dissolving in alcohol at 55°, and crystallizing out by cooling and adding a little water. The product is dried and stored in a glass-stoppered dark bottle in a cool place. Glassware used for the reagent must be free of all organic matter, hence it must be cleaned without soap and kept dust-free. Add 100 ml. of glacial acetic acid to 3 g. diphenylamine,

and, when completely dissolved, add 60 ml. cònc. hydrochloric acid, with stirring. Store in a glass-stoppered dark bottle in a cool place, and discard when the reagent begins to acquire a bluish color. Add a few crystals of sodium hyposulfite to improve the keeping quality.

Standard Glycogen Solutions. Prepare from glycogen reprecipitated from aqueous soln. by alcohol.

PROCEDURE

1. To the glycogen which has been centrifuged down and washed, add water to make a solution of suitable concentration—this will have to be determined for the particular case.
2. Add 5 vol. diphenylamine reagent to 2 vol. glycogen soln. Mix and centrifuge to eliminate any insoluble material. Avoid getting the reagent on the sides of the tube where it will evaporate during the heating and leave a film that will not go into solution.
3. Heat the tubes exactly 40 min. in boiling water and plunge into cold water for at least 3 min.
4. Run glycogen standards parallel with the unknowns.
5. Read color intensities (filter No. 635 with the Evelyn colorimeter) within 1 hr. after removal from the bath, using a water blank, and obtain the values of the unknowns from a calibration curve derived from the standards.

Van Wagtendonk, Simonsen, and Hackett Method for Glycogen

SPECIAL REAGENTS

Lugol Solution. Dissolve 1 g. iodine in a soln. containing 2 g. potassium iodide in 20 ml. water. Store in a well-stoppered dark bottle.

Standard Glycogen Solution. Dissolve 25 mg. glycogen (*Eastman Kodak Co.,* White Label) in 25 ml. 35% potassium hydroxide. (See note below.)

PROCEDURE

1. To a given vol. glycogen soln., diluted to an appropriate degree as determined in advance, add 0.01 vol. Lugol soln., and mix well.
2. Read the color at once (filter No. 54 with the Klett-Summerson colorimeter) using a blank consisting of the Lugol soln. diluted 100 times with water.

3. Obtain the results from a calibration curve derived by using various amounts of the standard glycogen soln. Subject the glycogen standards to the same steps of initial precipitation and color development as the unknowns.

NOTE: Constancy of temperature and iodine concentration are essential, and the glycogen standard should be prepared from glycogen obtained from the same source as the sample to be analyzed. See page 248.

VITAMIN A AND CAROTENE

By means of 2 mm. quartz microcuvettes and an adapter for the Beckman spectrophotometer (page 217), which was used for the absorption measurements, Bessey *et al.* (1946) succeeded in determining the vitamin A and carotene in as little as 35 μl. blood serum. Various volumes of serum greater than 35 μl. may be used as long as proportional volumes of the reagents are also employed. The procedure to be described is based on the use of 60 μl. The method involves saponification and extraction with solvents of low volatility, measurement of the absorption by the small volumes at 328 mμ (for vitamin A) and 460 mμ (for carotene), destruction of the vitamin A absorption by treatment with ultraviolet irradiation, and finally remeasurement of the absorption of 328 mμ. By measurement of the absorption of 328 mμ before and after the destruction of the vitamin A, absorption at this wavelength due to other substances will not interfere with the determination.

Method of Bessey *et al.* for Vitamin A and Carotene

SPECIAL APPARATUS

Ultraviolet Apparatus. A diagram of the apparatus used for the ultraviolet irradiation is shown in Figure 81. A mercury discharge lamp (General Electric B-H4) with purple envelope-filter and transformer are used to furnish the radiation. The brightest part of the lamp is placed opposite to the lower half of the tube, and the shadow of the electrode support is not allowed to fall on any tube. The tubes must be cooled during the irradiation by a moderate air current from a fan.

Mixing Apparatus. The device for mixing the liquids in the narrow tubes is made by cutting off the head of an eight-penny nail,

slightly flattening the end for a distance of 10–15 mm., inserting the nail in a small high-speed hand drill with the end projecting about 20 mm., and mounting the drill vertically with the nail up. The liquids are mixed by touching the side of the tube near the bottom to the rapidly rotating nail. If this apparatus is not available mixing may be effected by adding a 1 cm. length of stainless steel wire (0.041 in. diameter—may be obtained from *Newark Wire Cloth Co.*) and shaking. For the extraction with the kerosene-xylol, the open ends of the tubes are sealed in a flame and then shaken vigorously. Care is taken to prevent contamination of the tops of the tubes by serum which would be charred when the tubes are sealed.

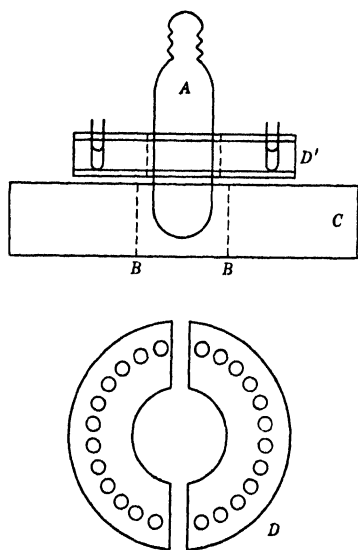



Fig. 81. Arrangement for ultraviolet irradiation. Mercury lamp (*A*) is held vertically in a clamp, base up, with the other end extending 3 or 4 cm. into a hole 8 cm. in diameter, *B–B*, in a large block of wood, *C*, which serves as a base. Semicircular racks (*D, D'*) are provided for holding the glass tubes in a circle equidistant from the lamp (6 cm. from the center of the lamp). These racks may be made from pieces of quarter inch plywood held about 2 cm. apart, with the upper piece drilled to hold the tubes. Twenty or thirty holes may be drilled in each rack along a semicircular line. *From Bessey et al. (1946)*

SPECIAL REAGENTS

1 N Potassium Hydroxide in 90% Alcohol. Add 1 vol. 11 *N* potassium hydroxide to 10 vol. absolute alcohol. Prepare on the day it is used. If the color develops rapidly or if the reagent gives a blank, reflux the alcohol with potassium hydroxide and distill.

Kerosene-Xylol. Mix equal vol. xylol, C. P., and water white odorless kerosene (obtainable from *Eimer and Amend*).
Anhydrous Propionic Acid.

PROCEDURE

1. Place 60 μl. serum and 60 μl. alcoholic potassium hydroxide in a test tube 100 × 3 mm. (Prepare the tubes by cutting 200 mm. lengths of glass tubing, 3–3.5 mm. internal diameter, cleaning with boiling half-conc. nitric acid, rinsing, drying, and dividing in the middle with a blast lamp flame to yield two tubes.) If the liquids do not run to the bottom, send them down with a whipping motion.
2. Mix the liquids, immerse the tube in a 60° water bath for 20 min., cool, and add 60 μl. kerosene-xylol.
3. Extract by holding the tube at a 45° angle against the whirling nail so that the contents are violently agitated for 10–15 sec. Then, when the tube is at room temperature or a little below, centrifuge for 10 min. at 3000 R.P.M.
4. Cut the tube with a file just above the kerosene-xylol layer and pipette this layer into the cuvette with a constriction pipette, taking care to avoid the inclusion of any of the aqueous liquid which would cause turbidity. (Use an uncalibrated 50–60 μl. constriction pipette (page 172) with a fine tip and a fine constriction because of the low surface tension of the organic solvents.)
5. Read the absorption at 460 and 328 mμ, and then transfer the liquid to a soft glass test tube 40 mm. long and 2.5–3.0 mm. internal diameter.
6. Irradiate with ultraviolet for 30–60 min. (Find the proper time by testing with known vitamin A solns. The time should be six to eight times that required to destroy half the vitamin in pure soln.)
7. Transfer the liquid back into a cuvette and take a second reading at 328 mμ. Rinse the pipette with anhydrous propionic acid before the transfer to eliminate traces of moisture which would cause turbidity.

CALCULATION

$$E_{460} \times 480 = \text{microgram per cent carotene}$$

$$(E_{328} - E_{328} \text{ after irradiation}) \times 637 = \text{microgram per cent vitamin A}$$

where E = optical density for 1 cm. cuvette = 2 − log per cent transmission with 1 cm. cuvette. The factor 637 is based on an E (1%, 1 cm.) of 1720 for vitamin A palmitate in alcohol at 328 mμ, calculated as free alcohol. The factor 480 is based on an E (1%, 1 cm.) of 2080 for β-carotene (*Smaco*) in kerosene-xylol.

III. TITRIMETRIC TECHNIQUES

A. MICROLITER BURETTES

The microliter burettes employed in histochemical procedures fall into two general groups. In one a capillary glass tube is calibrated so that the volume of liquid delivered can be determined by observing the position of a meniscus. These burettes are usually modifications of the Brandt-Rehberg (1925) instrument, which is arranged to move the column of solution by the pressure of a mercury thread controlled by a screw. Mercury in a reservoir is displaced by turning in the screw and the displaced mercury moves into the glass capillary. Instruments of this general type have been described by Pincussen (1927), Linderstrøm-Lang and Holter (1931, 1933a), Kirk (1933), Sisco, Cunningham, and Kirk (1941), Links (1934), and Boell (1945). In the Heatley (1935, 1939) microburettes the pressure is supplied by leveling-bulb arrangements, and both Conway (1934) and Hawes and Skavinski (1942) employ hydrostatic pressure in their instruments. The Conway burette was modified by Ramsay (1944) for use under anaerobic conditions (page 279).

In the other general group of burettes a calibrated capillary tube is not used, but the screw, usually in the form of a micrometer, is calibrated instead. These are essentially modifications of the instrument described by Widmark and Örskov (1928). Krogh and Keys (1931), Kirk (1933), and Krogh (1935) employed a fine screw to move the plunger of a small glass syringe for the accurate delivery of small volumes of liquid (page 174). Trevan (1925), Dean and Fetcher (1942), and Hadfield (1942) used the spindle of a micrometer to operate the plunger. Probably the best micrometer burette is that designed by Scholander (1942) and later improved by Scholander, Edwards, and Irving (1943). In this instrument the spindle of the micrometer is used to displace the mercury in the reservoir. An

advantage of this group of microburettes is that their accuracy is independent of the lumen of the capillary.

Linderstrøm-Lang and Holter Burettes. These instruments possess an approximately fivefold refinement of the original Brandt-Rehberg (1925) instrument, and they have been constructed in two main forms. The type 1 burette (Linderstrøm-Lang and Holter, 1931), shown in Figure 82, has a calibrated glass capillary tube

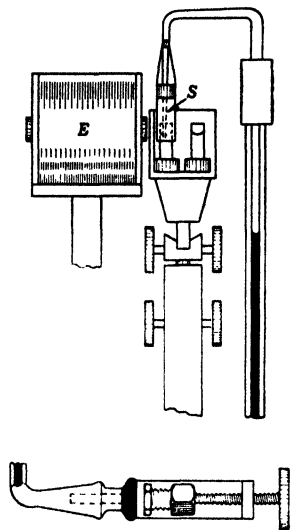



Fig. 82. Burette, type 1. *From Linderstrøm-Lang and Holter (1931)*

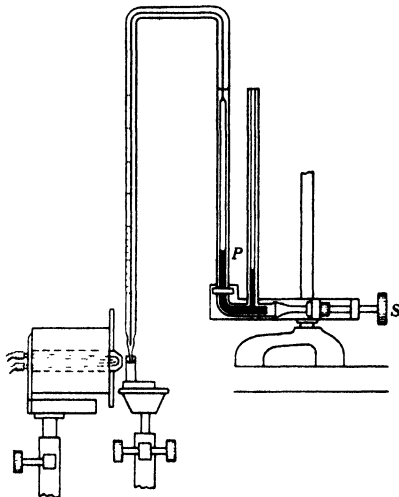



Fig. 83. Burette, type 2. *From Linderstrøm-Lang and Holter (1933a)*

58 cm. long, having a total capacity of 100 μl. and graduated in divisions of 0.2 μl. Estimations may be made to 0.02 μl. When the screw in the bottom is turned in, the mercury is forced up into the capillary, which, in turn, forces the liquid out of the burette. The tip of the burette is dipped into the liquid to be titrated in order that quantities less than a drop may be added. Readings are taken from the meniscus of the top of the mercury column. In filling the burette the tip is dipped into the standard solution and the screw is reversed. The top of the mercury column is in contact with the standard solution.

In the type 2 (Linderstrøm-Lang and Holter, 1933a), shown in Figure 83, the mercury is separated from the standard solution by an air space. This instrument is used for solutions which might be affected by contact with mercury. When the screw *S* is manipulated, the right mercury column, which is open to the air, is raised or lowered, and this results in a small positive or negative pressure over the left column. In this way liquid can be delivered from, or drawn into, the burette.

The type 1 burette can be connected to a permanent reservoir of standard solution as shown in Figure 84 (Linderstrøm-Lang and Holter, 1933b).

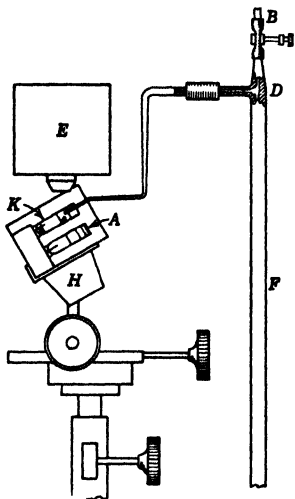



Fig. 8. Burette, type 1 with reservoir.
From Linderstrøm-Lang and Holter (1933b)

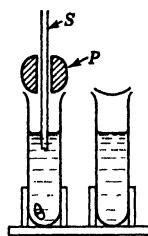



Fig. 85. Glass bead used to exclude air during titration.
From Linderstrøm-Lang, Weil and Holter (1935)

Reduction of evaporation and protection from the air during titration is afforded by the loosely fitting glass cap around the tip of the burette held suspended by two threads (Fig. 82). This effect may also be obtained by passing the tip of the burette through a glass bead, *P* (Fig. 85), which rests on the top of the titration vessel (Linderstrøm-Lang, Weil, and Holter, 1935). The glass bead may be dipped into paraffin oil first in order to effect a better seal to the titration tube. In order to carry out titrations in an atmosphere free

from carbon dioxide, Schmidt-Nielsen (1942) designed a soda lime container that fits on the titration tube as shown in Figure 86.

It may be observed in Figures 64 and 84 that a titration table is employed which is adjustable both vertically and horizontally. An opal-glass background, illuminated by a small electric bulb, is

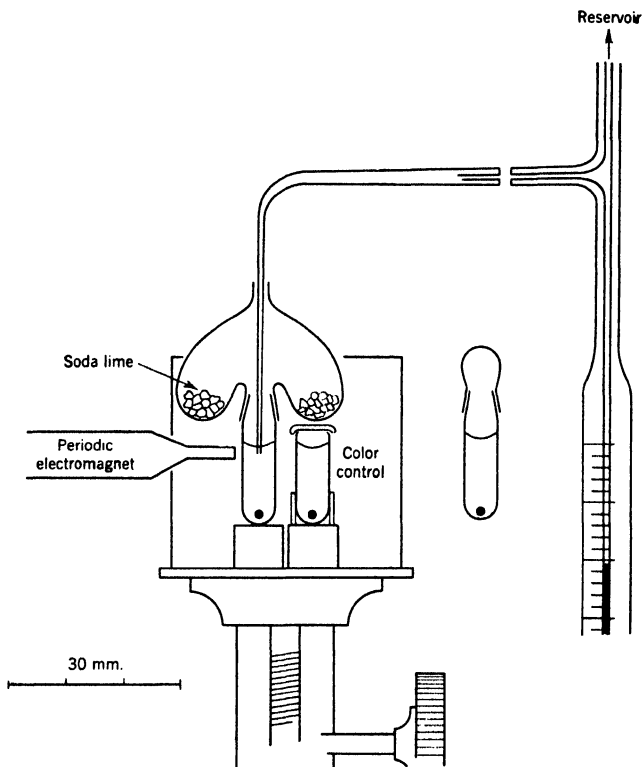



Fig. 86. Titration with "desiccator" to maintain carbon-dioxide-free atmosphere.
From Schmidt-Nielsen (1942)

provided to facilitate the observation of the color of the solution. The electromagnet placed to the left of the titration table is used for magnetic stirring in the manner described in the section dealing with stirring devices (page 179).

The complete titration assembly is available from *A. H. Thomas Co. and* E. Petersen, Carlsberg Laboratory.

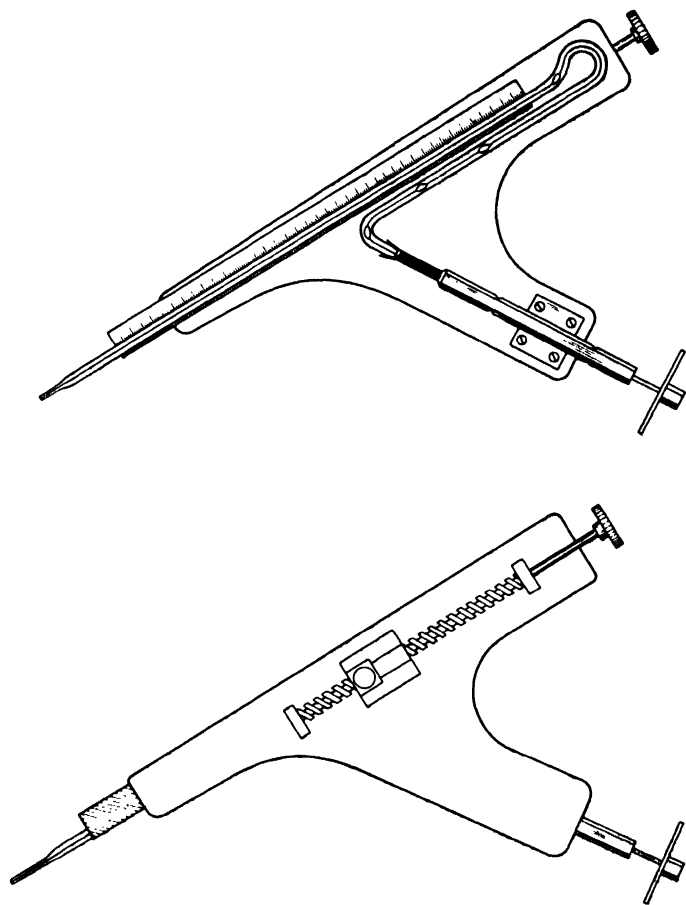



Fig. 87. Burette, front and rear views.
From Sisco, Cunningham, and Kirk (1941)

Kirk Burette. In this instrument (Fig. 87) the mercury is separated from the standard solution by air, and the readings are taken from a scale behind the capillary tube rather than from

graduations on the tube itself. The burette has a total capacity of about 0.1 ml. and is capable of a precision in reading of ± 0.03 μl. (Sisco, Cunningham, and Kirk, 1941). (Available from *Microchemical Specialties Co.*)

Heatley Burette. The essential differences between the Heatley (1935) burette and the preceding models are that the mercury displacement is effected by means of a leveling bulb rather than a screw, the standard solution is in contact with paraffin oil, and delivery is made directly from a stock bottle of the solution. A diagram of the instrument is shown in Figure 88. The tube leading

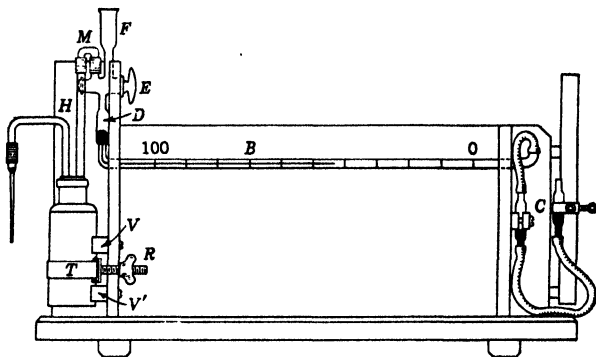



Fig. 88. Burette.
From Heatley (1935)

from the delivery tip extends to the bottom of the stock bottle which has a 2 oz. capacity and is lined with paraffin. The stopper of the stock bottle is a cork infiltrated with paraffin, and the 5 mm. vertical tube *H* ends flush with the bottom of the cork. The upper part of *H*, the capillary connection (*M*), the three-way stopcock (*E*), reservoir (*F*), and the space (*D*) are all filled with paraffin oil. The space under *D* and part of the capillary tube (*B*) contain mercury. The leveling bulb arrangement (*C*), by which the pressure is regulated, also contains mercury. An air space exists between the leveling bulbs and the mercury thread in the capillary. No air is permitted between the mercury under *D* and the delivery tip. Interchangeable delivery tips fitted through a ground-glass joint may be used. When not in use, the tip is dipped into a tube of the titration solution

covered by a layer of paraffin oil. This serves to protect the tip, and if the protecting tube and the tip are sealed together by a rubber connection, siphoning-over of the solution is prevented. By adjusting *C* so that only a small positive pressure is applied, the surface tension at the fine tip will prevent liquid from escaping, and delivery will occur only when the tip is immersed in the solution to be titrated. This, of course, is the principle used for the other microburettes that have been described. Stock bottles can be interchanged without affecting the capillary in any way. One of the instruments that Heatley constructed had a capacity of 0.1 ml. over a 25 cm. scale, and the capillary was divided in 1.0 μl. graduations. The relatively large volume of the stock bottle and the air space between the mercury in the leveling bulb and that in the capillary would make this burette particularly prone to errors arising from temperature fluctuations during titration.

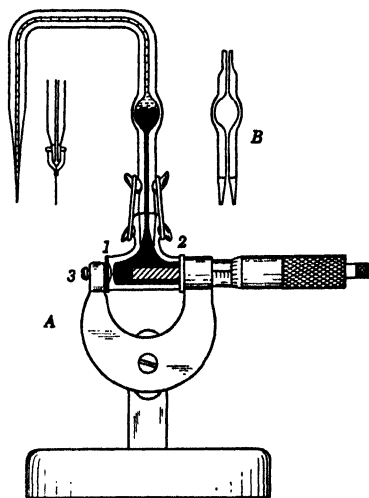



Fig. 89. Micrometer burette.
From Scholander, Edwards, and Irving (1943)

Boell Burette. One of the easiest burettes to construct is that of Boell (1945). The instrument consists of a horizontal calibrated capillary tube drawn out to a fine delivery tip at one end, bent vertically and pointing downward; the other end of the capillary is

connected to a rubber tube that acts as a mercury reservoir. One end of the rubber tube is plugged to retain the mercury, and a screw clamp is used to force the mercury out of, or to draw it into, the reservoir. An air space is left between the mercury and the titration liquid. While lacking some of the refinements of other models, it can be made into a serviceable instrument.

Scholander Burette. A diagram of this instrument is shown in Figure 89; an all-steel micrometer, with its anvil removed, is used. A burette (*A*) for titration, or a burette (*B*) for calibration of pipettes and syringes may be fitted by a ground-glass joint to the chamber containing the micrometer spindle. The volume of the bulbs in the burettes should approximate the volume that the spindle can displace. A medium-heavy grease is applied to the micrometer spindle, and the spindle chamber is fixed in position by means of a set screw (*3*) which presses against the steel disc (*1*) having a recessed punch mark; a lightly greased paper or fiber gasket (*2*) seals the open ground end of the chamber held against the spindle bearing. The gasket should fit the spindle tightly. With the spindle retracted until flush with the bearing face, the chamber is filled with mercury through the open ground socket, taking care to remove all air bubbles. The air bubbles adhering to the walls can be removed by touching the bubbles with the end of a fine steel wire and leading them out. Bubbles at the ground socket are avoided by placing several drops of water or titration liquid over the mercury in the socket before inserting the upper part of the burette. Extra mercury can be drawn in through the tip, if necessary. The spindle chamber should be made as small as required to just clear the spindle. By keeping the chamber volume small, and with proper handling of the instrument, temperature errors can be reduced to the point where a water jacket is not necessary.

The micrometer employed has a total spindle excursion of 25 mm. marked off in 2500 scale divisions. Estimations are made to one fifth of a division. Calibration may be carried out by weighing delivered quantities of water, and relating their volumes to the number of scale divisions required to deliver them. The total capacity of the burette can be delivered with an accuracy of 1 part in 6000 to 7000. With the ordinary spindle, volumes can be measured with an over-all accuracy of about 0.1 μl. By replacing the spindle with a $^1/_{16}$ in. drill rod, a refined burette can be constructed capable of measuring

delivered amounts with an accuracy of about 0.02 μl. The burettes are commercially available from *Emil Greiner Co.* and from O. Hebel, Edward Martin Biological Laboratory, Swarthmore College.

Loscalzo and Benedetti-Pichler Burette. Loscalzo and Benedetti-Pichler (1945) described the titration of microgram samples in volumes of 0.05–0.50 μl. using a burette (Fig. 90) consisting of

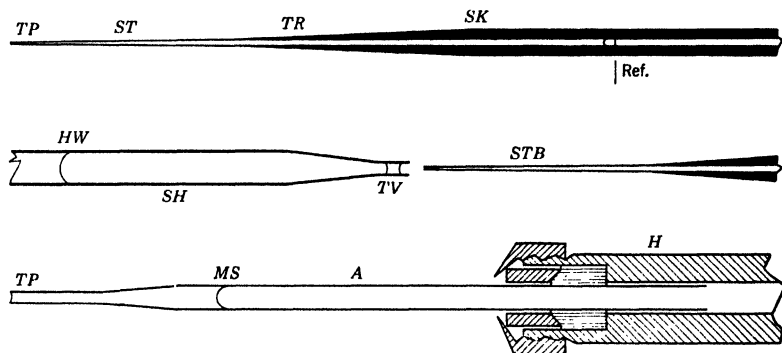



Fig. 90. Titration of microgram samples. Upper drawing shows burette with remote control, 7× natural size. Center drawing shows open titration cone, 7× natural size. At bottom, burette, schematic: *TP*, tip; *ST*, shaft; *TR*, taper; *SK*, shank; Ref., reference mark; *SH*, shank of titration cone; *TV*, titration cone proper; *STB*, shaft of burette; *HW*, meniscus of hydraulic water; *MS*, of standard solution; *A*, air column; *H*, pipette holder of metal with rubber washer (horizontal shading. *From Loscalzo and Benedetti-Pichler (1945)*

a piece of capillary tubing drawn out to a fine tip. The titrations are carried out in a moist chamber on the stage of a low-power microscope and the operations are controlled by a micro manipulator. Measurements of the quantity of the solution delivered from the burette are made by observing the displacements of the meniscus on the scale of an eyepiece micrometer used with the microscope. The solution is moved by the pressure transmitted from a leveling-bulb arrangement (Fig. 91). The method of handling the small volumes of liquid was developed by Benedetti-Pichler (1937) for micro qualitative analysis, and it was expanded later by Benedetti-Pichler and Rachele (1940), and Benedetti-Pichler and Cefola (1942). Details of the apparatus and manipulations will not be given since there have been no histochemical applications of titrations in volumes of 0.05–0.50 μl. However, the possibilities in this respect are somewhat intriguing.

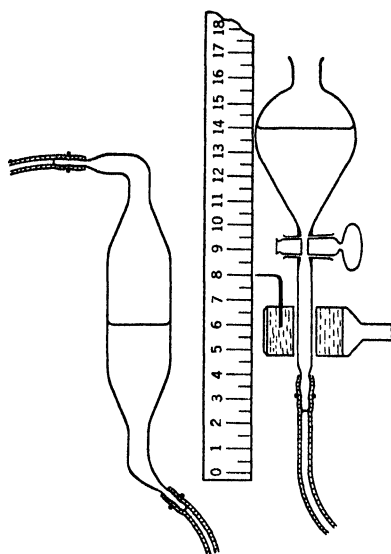



Fig. 91. Leveling bulbs for use with micropipettes. *From Loscalzo and Benedetti-Pichler (1945)*

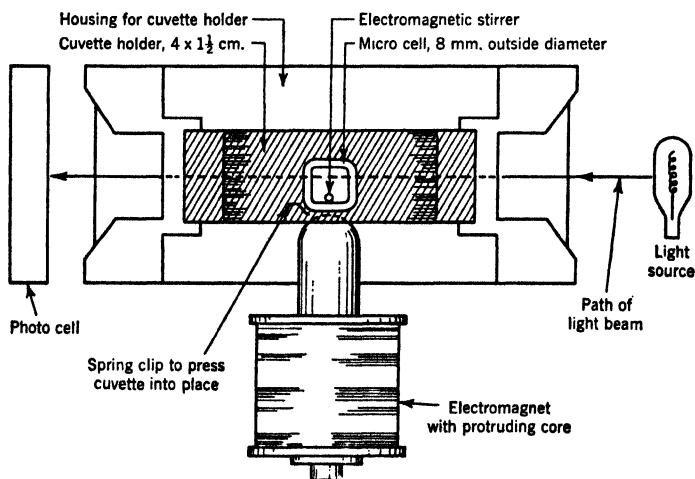



Fig. 92. Detailed diagram of microcuvette in holder, showing relationship of electromagnetic stirrer to light beam. *From Zamecnik, Lavin, and Bergmann (1945)*

B. PHOTOMETRIC END POINTS

For the objective determination of end points in micro titrations, Zamecnik, Lavin, and Bergmann (1945) employed a photoelectric apparatus. A Pfaltz and Bauer fluorophotometer (model A) was used, and the titration vessel was a square, 6 × 6 mm. ungraduated hemometer tube 3 cm. long. The tube was placed in an adapter which fitted into the cuvette holder and the titration was carried out with the vessel in the instrument (Fig. 92). Electromagnetic stirring was employed as shown. The end point was indicated by the galvanometer reading which corresponded to the maximum rate of decrease in light transmission per microliter of standard titration solution.

The apparatus was applied to the Linderstrøm-Lang acetone titration of amino groups using naphthyl red as the indicator. A Wratten filter 77 was used in order to obtain light in the region of 540–550 mμ for the photometry. Actually, the acetone titration can be performed satisfactorily by visually matching the color to the arbitrary end point color chosen for the blank (page 304), but there could be instances in which it would be of advantage to have an objectively fixed end point.

C. METHODS

SODIUM AND POTASSIUM (COMBINED)

A method has been described by Linderstrøm-Lang (1936) for the estimation of sodium plus potassium in small samples of biological material containing less than 4×10^{-4} milliequivalent of the alkalis with a precision of 1×10^{-6} milliequivalent. The determination involves ashing the sample with a reagent to convert the sodium and potassium to chlorides, removal of other chlorides, and electrometric titration of the residual chloride with silver nitrate. During the removal of extraneous chlorides by heating, a slight loss of sodium and potassium chloride occurs even when the temperature is held as low as 330°, the sublimation temperature of ammonium chloride. However, this loss is fairly reproducible and it is usually

sufficient to compensate for it by multiplying the result by the factor, 1.04.

From the results of various tests, it was shown that the ashing reagent removes all sulfate and phosphate; the presence of calcium, magnesium, and iron in the sample does not disturb the determination.

Linderstrøm-Lang Method for Sodium and Potassium

SPECIAL APPARATUS

Quartz Tubes. These are of clear quartz having an inner diameter of 3.8 mm., an outer diameter of 6 mm., and a length of 20 mm. The cleaning of the tubes requires a special procedure in this case: Completely fill with dil. (1 *N*) hydrochloric acid (to dissolve barium carbonate). Wash out and fill with 2 *N* ammonia (to dissolve silver chloride). Wash out, fill, and heat with conc. sulfuric acid (to dissolve barium sulfate), and finally wash several times with boiling water. In all operations, avoid touching the rim of the tubes with the fingers.

Incineration Oven. The oven, shown in Figure 65, is made of a solid copper block containing holes 18 mm. deep and about 7 mm. in diameter. Two electric heaters placed at the sides of the block enable a temperature of 440–460° to be maintained. A rheostat is used to obtain lower temperatures and to regulate the heating. The sides and bottom of the oven are insulated with asbestos.

Hand Pipette for Ashing Reagent (about 10 μl. capacity). Made of a piece of capillary tubing with a rather wide aperture to avoid plugging by barium carbonate, which forms during pipetting.

SPECIAL REAGENTS

Ashing Reagent. Prepare a soln. containing 1.2% barium chloride ($BaCl_2.2H_2O$) and 3.2% barium hydroxide ($Ba(OH)_2.8H_20$).

Precipitation Reagent. Dissolve 5.7 g. ammonium carbonate in 100 ml. 1.5 *N* ammonium hydroxide.

Titration Medium. Prepare a soln. containing 1.20% sodium nitrate and 0.40% secondary sodium phosphate ($Na_2HPO_4.2H_2O$) in 0.10 *N* nitric acid.

Silver Nitrate Solution (0.02 N). Dissolve 0.3398 g. silver nitrate in 100 ml. titration medium.

PROCEDURE

1. Transfer sample of tissue containing 1×10^{-5} to 4×10^{-4} milliequivalent of the alkalis into the bottom of a quartz tube. If necessary, about 8 μl. water can be placed in the tube to receive the tissue.
2. Add about 10 μl. ashing reagent with the simple hand pipette and place in an oven at 106° to evaporate the water.
3. When dry, place in the incineration oven at 440–460° for 30 min. The grayish-white ash may contain dark particles of carbon, but these do not influence the analysis.
4. Add 100 μl. distilled water to the ash with a calibrated constriction pipette, and stir carefully with a thin glass rod (1–2 mm. diameter.
5. Cap the tube and centrifuge for 5 min. at 2000 R.P.M.
6. Draw off 70–80 μl. of the supernatant with a calibrated constriction pipette and transfer to a glass reaction tube of the usual type (page 166).
7. Add 15 μl. precipitation reagent with another standardized constriction pipette and introduce a stirring "flea."
8. Cap the tube and let stand 2 hr., stirring the mixture from time to time; then remove the "flea" with the electromagnet.
9. Centrifuge as before and transfer 70–80 μl. supernatant to a quartz tube using the same pipette as previously.
10. Evaporate the water at 106° and incinerate for 1 hr. at 360°.
11. Add 50 μl. titration medium and titrate the chloride with the silver nitrate soln. using the electrometric procedure (page 282).
12. Run control experiments, omitting the sample, to obtain a correction for alkalis in the reagents; the titration value of these control runs should not exceed 0.2 μl. silver nitrate soln.
13. Standardize the silver nitrate soln. by carrying a known quantity of pure potassium chloride through the steps in the procedure.

NOTE: If in the above procedure it is assumed that pipettes having a capacity of 101.1, 73.0, and 15.2 μl., respectively, were employed the calculation becomes:

$$\text{Original content} = \text{titration value} \times \frac{101.1 \times 88.2}{73.0 \times 73.0}$$

POTASSIUM

Norberg (1937) developed a method for the determination of less than 1×10^{-4} milliequivalent of potassium (< 3.91 μg.) with a precision of about 1 to 2×10^{-6} milliequivalent in samples of biological material containing between 1×10^{-5} and 3.5×10^{-4} milliequivalent. The method is based on precipitation, as the chloroplatinate, of the potassium extracted from incinerated biological material, isolation of the precipitate by centrifugation, conversion of the chloroplatinate to the iodoplatinate, and titration of the latter with thiosulfate. Tests revealed that the presence of sodium in concentrations 150 times that of the potassium had no influence.

A method described by Cunningham, Kirk, and Brooks (1941) is suited to the analysis of biological material when the ratio of sodium to potassium does not exceed twenty. For quantities of potassium over 2 μg. the error does not exceed 0.5%, and over 0.7 μg. it does not exceed 3%. As in the Norberg method, the potassium extracted from the incinerated sample is precipitated as the chloroplatinate. Cunningham *et al.* collect the precipitate on a filter stick, dissolve the substance, reduce it with sodium formate, and titrate the chloride thus produced electrometrically.

Only the Norberg method will be described, since it has the advantage of employing a simple indicator titration rather than the electrometric procedure, and the separation of the chloroplatinate by centrifugation would also appear to be a little simpler than filtration on a specially constructed filter stick.

Norberg Method for Potassium

SPECIAL APPARATUS

Apparatus for Ashing the Sample. That employed in the combined sodium and potassium method (page 266) of Linderstrøm-Lang is used.

Glass Precipitation Tubes. These may be either of the forms shown in Figure 36 (page 167).

Equipment for Removal of Supernatant Fluid over the Precipitate. Illustrated in Figure 37 (page 167). Suction is applied at *A*

through a rubber tube. The low-power microscope aids in the careful removal of the liquid.

SPECIAL REAGENTS

Ashing Reagent. 1.2% barium chloride ($BaCl_2.2H_2O$) soln. containing 3.2% barium hydroxide ($Ba(OH)_2.8H_2O$).

Precipitation Reagent. Prepare 0.02 *M* chloroplatinic acid by dissolving 0.1 g. of the substance ($H_2PtCl_6.4H_2O$) in distilled water and making up to 10 ml. The soln. is stable for at least a month if stored in a refrigerator.

Pure Absolute Alcohol.

Phosphate Buffer (*p*H 6.98). Combine 4 ml. *M*/15 primary potassium phosphate, 6 ml. *M*/15 secondary sodium phosphate, and 90 ml. distilled water.

2 N Potassium Iodide. Dissolve 3.32 g. potassium iodide in water and make up to 10 ml. Filter if necessary. The soln. must contain no free iodine.

0.02 N Sodium Thiosulfate (0.496% $Na_2S_2O_3.5H_2O$). Standardize preferably by treating 7–8 µl. 0.01 *N* potassium chloride in the same fashion as the soln. of the unknown.

Green-Light Filter Solution. Add 4 ml. *M*/15 phosphate buffer, *p*H 6.36 (7.5 ml. primary potassium phosphate + 2.5 ml. secondary sodium phosphate) to 6 ml. 0.04% bromothymol blue. Do not use the soln. longer than a week, since the color eventually changes.

PROCEDURE

1. Ash the sample as described in the combined sodium and potassium method of Linderstrøm-Lang (page 267) and extract the ash with 100 µl. water in the same manner.

2. Pipette an aliquot of about 100 µl. of the centrifuged clear extract containing 1×10^{-5} to 3.5×10^{-4} milliequivalent potassium into a precipitation tube and add 10 µl. precipitation reagent with a hand pipette.

3. Evaporate the water at 90–95°; it does not matter if the heating continues for 1–2 hr. after the water has been driven off.

4. Wash the residue with 100 µl. absolute alcohol, added with a hand pipette. Use a platinum needle 0.8 mm. thick to stir up the solid and rinse the needle with 10 µl. absolute alcohol before removing it from the tube.

5. Cap the tube with a short piece of clean rubber tubing plugged at one end with a glass ball. The rubber should have no rough surfaces which might rub off and contaminate the precipitate with particles. Centrifuge for 5 min. at 2000 R.P.M. (radius 14 cm.).

6. Draw off the supernatant liquid with the capillary tube arrangement leaving less than 5 μl. covering the bottom of the tube to a depth of about 1 mm.

7. Repeat the washing process and dry the precipitate by heating at 95° for 30 min.

8. Add 45 μl. boiling hot phosphate buffer, *p*H 6.98, with a hand pipette to dissolve the precipitate. Cap the tube and set aside for 6–12 hr. to insure complete soln.

9. Add 15 μl. 2 *N* potassium iodide with a hand pipette, introduce a stirring "flea," cap the tube, and mix the contents.

10. After 30 min. and up to 24 hr., titrate the liquid with 0.02 *N* thiosulfate to the disappearance of the rose color. Carry out the titration in the green light obtained by passing the light from an electric bulb fixed on the back of the titration stand through a cell filled with the green-light filter soln. Compare the end point color to that of the tube of water placed beside the titration tube.

SODIUM

Lindner and Kirk (1938) described a method for the determination of sodium which can be applied to small samples containing 0.13–4.13 μg. sodium. The standard error, on the average, is reported as a few tenths of a per cent. The method depends on the precipitation of sodium as sodium zinc uranyl acetate, isolation of the precipitate, reduction of the uranium with cadmium, and titration of the reduced uranium with ceric sulfate. The procedure involves several quantitative transfers, not of aliquots, but of total material. Hence scrupulous care must be exercised to avoid loss at each of these steps (see page 165).

The procedure of Clark *et al.* (1942) might be adapted to the required micro level for histochemical work. In their procedure, the sodium zinc uranyl acetate is simply dissolved and titrated with sodium hydroxide using phenolphthalein as indicator. Each sodium atom in the sodium zinc uranyl acetate is equivalent to nine of the alkali molecules.

. Sodium may also be determined by difference using both the Linderstrøm-Lang method for sodium plus potassium (page 266) and the Norberg method for potassium alone (page 268). A colorimetric method for sodium is given on page 203.

Lindner and Kirk Method for Sodium

SPECIAL REAGENTS

Calcium Hydroxide.

0.1 N Hydrochloric Acid.

Zinc Uranyl Acetate Solution. Prepare as described on page 203 and let stand 24 hr. Filter through a sintered-glass bacteria-proof filter. Should turbidity appear, refilter.

Wash Solution. Saturate 95% alcohol with sodium zinc uranyl acetate and filter absolutely clear as above.

Asbestos. Grind washed and ignited Italian asbestos in a mortar until fine. Boil in successive portions of ceric sulfate soln. acidified with sulfuric acid. Wash well with water and store in an all-glass container.

5% Sulfuric Acid. Prepare from acid redistilled in a glass apparatus.

0.01 N Ceric Sulfate Solution. Standardize against pure sodium oxalate or potassium ferrocyanide.

Indicator Solution. 0.0025 *M* phenanthroline ferrous sulfate or 0.1% setopaline C (see page 275).

Standard Sodium Chloride Solutions.

Spirals of Cadmium. Cut a thin ribbon from a stick of the metal with a lathe tool. Flatten the helix formed to a disc having a total surface of about 2 $cm.^2$; leave one end extending upward to serve as a handle. This reductor may be used almost indefinitely.

Water. Redistill all water used from an all-glass apparatus.

PROCEDURE

1. Incinerate the sample at about 450° for several hr. in a small platinum crucible.

2. Add about 50 μl. 0.1 *N* hydrochloric acid to the cooled clean white ash.

3. Transfer with washing by means of a large capillary pipette to a centrifuge cone of about 0.2 ml. capacity. Add a little calcium hydroxide with a toothpick and mix well to precipitate phosphate.

4. After standing 2–4 hr., add a drop of water and centrifuge for at least 5 min. at about 5000 R.P.M.

5. Transfer the supernatant quantitatively, with washing of the precipitate, to a porcelain titration dish (Fig. 44, page 169) using a capillary pipette.

6. Evaporate to a small vol. just short of dryness, and while still warm, add 1 ml. zinc uranyl acetate reagent. Stir and warm for about 5 min.

7. Cover with a Petri dish and let stand 12–24 hr. for complete crystallization. If reagent crystallizes during this period, place a container of water under the Petri dish to prevent evaporation from the sample.

8. Suck off the supernatant through a sintered-glass filter stick covered with fine asbestos (Fig. 61). Wash the porcelain dish and precipitate thoroughly with the alcoholic wash soln.

9. Remove the asbestos to the porcelain dish with a drop of 5% sulfuric acid, which is also used to wash the end of the filter. Rinse with a little water.

10. Insert the cadmium spiral, warm and stir for 5 min.; then remove and thoroughly wash the cadmium with water.

11. After cooling for 5 min., titrate the reduced uranium with ceric sulfate using one of the indicators.

12. Run a blank determination on the reagents by repeating the entire procedure without a sodium sample.

CALCIUM

Two methods for the microestimation of calcium have been given by Siwe (1935b) and Lindner and Kirk (1937a) have described still another. All the methods employ precipitation of calcium as the oxalate. In one of Siwe's methods the precipitate is dissolved in acid, an excess of permanganate is added followed by potassium iodide, and the iodine liberated by the excess permanganate is titrated with thiosulfate. In the other method the oxalate is converted to carbonate by heating, an excess of acid is added, and the excess titrated with alkali. The Lindner and Kirk procedure uses an excess of ceric sulfate to react with the oxalate dissolved in acid, followed by titration of the excess with ferrous ammonium sulfate. Kirk and Tompkins (1941) compared oxalate titrations by different methods and

found that the ceric sulfate procedure was superior to the direct permanganate titration when the latter is used with setopaline C to sharpen the end point. However, no comparison was made with the iodometric permanganate method as used by Siwe. Siwe employed his methods for measurements of the calcium in 50 μl. samples of serum and reported a precision of ±3.5% for both procedures. Lindner and Kirk stated that their method was adapted to the determination of 0.5–12 μg. calcium with a precision of less than ±0.5% on all but the lowest amounts of calcium.

The procedure followed by Sobel and Kaye (1940), which substitutes an iodometric for the acidimetric titration of excess acid in the Siwe method, could be adapted to histochemical work. This involves ignition of the calcium oxalate precipitate to convert it to the carbonate, solution of the carbonate in an excess of acid, and iodometric determination of the excess acid by addition of an excess of potassium iodate and potassium iodide, followed by titration of the liberated iodine with thiosulfate. By employing very dilute thiosulfate (0.0007 *N*) as little as 4 μg. calcium could be measured using a 5 ml. burette for the titration. By carrying out the oxalate precipitation at *p*H 3.0 to 3.5, the authors minimized coprecipitation of magnesium. Conversion of the oxalate to carbonate was considered advantageous since the oxalate precipitate could then be washed with ammonium oxalate to reduce the loss during the washing, and any excess of oxalate ion in the precipitate would be volatilized in the ignition. See page 219 for a colorimetric method.

Siwe Iodometric Permanganate Method for Calcium

SPECIAL REAGENTS

Ammonium Hydroxide (conc.).
5% Ammonium Oxalate.
25% Nitric Acid.
1% Potassium Iodide.
0.2% Soluble Starch.
0.01 N Potassium Permanganate.
0.01 N Sodium Thiosulfate.

PROCEDURE

1. Place a soln. of the sample having a vol. of about 50 μl. into

a reaction tube, add a very small drop of ammonium hydroxide, and drop in a mixing "flea."

2. Warm to about 50–60°, mix well, and add 50 μl. ammonium oxalate soln. Let stand 2–6 hr.

3. Centrifuge, remove the supernatant with a pipette, and wash the precipitate twice with 100 μl. portions of ice-cold water.

4. Centrifuge 15–30 min. and pipette off the supernatant.

5. Dissolve the precipitate in 25 μl. of the nitric acid by stirring and heating for 30 sec. in boiling water.

6. Add 50 μl. permanganate soln., and after 1–1.5 min. add 25 μl. iodide soln.

7. Titrate the iodine liberated with thiosulfate until the yellow color is almost gone. Then add a very small amount of starch soln. and continue the titration until the blue color just disappears.

8. Run a control by titrating as above but omitting oxalate from the mixture.

NOTE: It has been the experience of the Carlsberg Laboratory workers that a serious loss of iodine can occur by evaporation in iodometric methods unless a liquid seal is placed across the lumen of the reaction tube. Therefore it would be advisable to follow the technique given on page 311, steps 4–5.

Siwe Acidimetric Method for Calcium

SPECIAL REAGENTS

Ammonium Hydroxide (conc.).
5% Ammonium Oxalate.
0.01 N Hydrochloric Acid.
0.01 N Sodium Hydroxide.
1% Phenolphthalein.

PROCEDURE

1–4. Same as steps in the preceding permanganate method. Use a heat-resistant glass reaction tube.

5. Heat on a sand bath or in an oven at 550–600°, not exceeding the latter, for 30 min.

6. After cooling, pipette in 50 μl. 0.01 *N* hydrochloric acid and heat for 30 sec. in boiling water.

7. Add a very small amount of phenolphthalein and titrate with 0.01 *N* sodium hydroxide to the first pink color.

8. Run a control by titrating 50 μl. of the acid with the alkali to the same end point.

NOTE: The precautions given on page 257 for the exclusion of atmospheric carbon dioxide during the titration should be followed. It would also be advantageous to substitute tetramethylammonium hydroxide for the sodium hydroxide in order to avoid the difficulties occasioned by the use of the latter in fine-bore microburettes; thymol blue will serve as a better indicator than phenolphthalein (page 291).

Lindner and Kirk Cerimetric Method for Calcium

SPECIAL REAGENTS

Water. Distill all water used for reagents or otherwise from an all-glass apparatus, and likewise distill all sulfuric acid used.

4% Ammonium Oxalate. Filter through fine asbestos on a sintered-glass filter.

Wash Solution. Saturate 10% (by vol.) ammonium hydroxide with freshly precipitated and washed calcium oxalate. Filter through asbestos on a sintered-glass filter. Prepare fresh from time to time to avoid the formation on long standing of a very fine suspension that cannot be properly filtered.

Saturated Sodium Acetate.

0.01 N Ceric Sulfate.

0.01 N Ferrous Ammonium Sulfate in 0.1 N Sulfuric Acid. Store in a dark bottle under illuminating gas and withdraw portions as needed. The soln. is stable in contact with air for 2 weeks or longer.

0.0025 M Phenanthroline Ferrous Sulfate. Prepare from a stock soln. of 0.025 *M* which is made up by dissolving 1.485 g. *o*-phenanthroline in 100 ml. of 0.025 *M* ferrous sulfate.

The advantage of using nitro-*o*-phenanthroline ferrous sulfate has been emphasized by Salomon *et al.* (1946). A 0.05% soln. of lisamine green (*British Drug House Ltd.*) gives a smaller blank than the o-phenanthroline ferrous sulfate, according to Nimmo-Smith (1946), who also recommends the latter reagent because it is cheaper.

0.1 N Hydrochloric Acid.

6 N Sulfuric Acid.

Asbestos. Treat all asbestos used as follows: Wash and ignite Italian medium fiber asbestos, and grind in a mortar. Boil in acidified ceric sulfate soln. and wash well with water on a sintered glass filter. Store a suspension of the material in water.

PROCEDURE

1. If sample must be ashed, place in a small platinum crucible and heat in a micro furnace not exceeding 450°. Dissolve ash in a drop of 0.1 *N* hydrochloric acid and transfer the liquid to a porcelain titration dish (Fig. 44, page 169) using a pipette. Rinse crucible and pipette with water to make the transfer quantitative (see page 165).
2. Warm the acidified soln. of the sample in the titration dish, insert a thread stirrer, and add, with mixing, a vol. of 4% oxalate soln. equal to that of the sample.
3. Add 0.1 ml. saturated sodium acetate and continue heating for 5 min., adding a little water to keep up the vol.
4. Let stand overnight protected from dust.
5. Filter the calcium oxalate with a micro external filter stick (1.5 mm. diameter, and covered with a thin layer of asbestos). Use about 30 drops of wash soln. to rinse the dish and the precipitate on the filter (see page 177).
6. Transfer asbestos pad to titration dish by using a drop of 6 *N* sulfuric acid. Wash the filter with a few drops of the sulfuric acid followed by a few drops of water.
7. Heat the dish for 2 min. while stirring to dissolve the oxalate, and then let cool for 5 min.
8. Add a measured excess of standard ceric sulfate, stir for 3 min. and add 5 μl. of the phenanthroline indicator. Titrate the excess with ferrous ammonium sulfate.
9. Run a control on the reagents alone.

IRON

Kirk and Bentley (1936) developed a micro method for the estimation of iron which involves the reduction of the iron by cadmium amalgam, addition of a measured excess of ceric sulfate, and titration with ferrous ammonium sulfate using phenanthroline ferrous sulfate as the indicator. The method is adapted to the measurement of 2–15 μg. iron. With blood or simple solutions a mean error of 1–2%, and a maximum error of 4%, has been reported.

Ramsay (1944) reported that in his laboratory all reductions with pure metals and amalgams gave blanks amounting to several micrograms of iron. Accordingly, he employed a titanometric method. Small amounts of copper have been found to interfere with the

analysis, but Ramsay succeeded in circumventing this difficulty by a selective extraction of the iron. As described, the method may be used for the determination of 10–200 μg. iron. When applied to analysis of 0.2 ml. portions of blood, the coefficient of variation was 0.45%. A bother characteristic of the titanometric method is that frequent standardization is required and the titration must be carried out in a manner which will prevent access of oxygen to the solution; furthermore, the standard solution must be protected from oxygen at all times. Ramsay employed a horizontal burette arranged as shown in Figure 93. The standard solution is kept in contact with an atmosphere of hydrogen, and a stream of carbon dioxide bubbles is used for stirring.

With the development of microcuvettes, the application of the colorimetric methods for iron, *e.g.*, the α,α'-dipyridyl or thiocyanate reactions, to the small quantities considered in histochemical work may be expected to offer more sensitive analyses, and possibly by simpler procedures.

Kirk and Bentley Method for Iron

SPECIAL REAGENTS

5% Sulfuric Acid (iron free).

3% Cadmium Amalgam. Dissolve 3 g. pure cadmium in 100 g. mercury and store in a well-stoppered, deep, narrow vessel to minimize air oxidation.

0.01 N Ceric Sulfate.

0.01 N Ferrous Ammonium Sulfate. Store as indicated on page 275.

0.025 M Phenanthroline Ferrous Sulfate. Dilute ten times before using (page 275).

PROCEDURE

1. If the sample requires ashing, place in a platinum crucible (8 mm. deep, 12 mm. top diameter) and heat for 10 min. just below visible redness. The small muffle furnace described on page 180 is very well suited to the purpose. Heating at too high a temperature or for too long may cause formation of insoluble ferric oxide. The iron concentration in blood is so high that a five fold dilution is necessary to obtain a convenient sample (3–15 μg. iron). Addition of some iron-free sodium hypochlorite soln. to the water used in the dilution serves to dissolve clot fragments and assist in the ashing. Drive off

the water by heating on a hot plate before proceeding to the ashing.

2. Dissolve the ash in 5% sulfuric acid, and, if the insoluble form of ferric oxide is present, remove the acid extract and heat the moist residue on a hot plate until sulfur trioxide fumes appear. Then dissolve the residue in 5% sulfuric acid and add the liquid to the first extract.

3. Pipette soln. of sample into a microreductor (Fig. 48, page 171) and adjust acidity so that when diluted to vol. the acid concentration will be 0.1 *N*. Usually 40–75 μl. 5% sulfuric acid is required for solns. of ashed samples.

4. After the vol. is made up to the mark, add 30 μl. 3% cadmium amalgam; stopper and shake at intervals or continuously for 10 min.

5. Pipette about twice the vol. of 0.01 *N* ceric sulfate needed to react with the unknown into a dish (Fig. 44, page 169). Add a measured quantity of indicator soln. and start the thread stirrer.

6. Open the reductor and pipette an aliquot into the ceric sulfate soln.

7. Titrate with ferrous ammonium sulfate to a permanent reddish tint.

8. Run a control by titrating the ceric sulfate without a sample.

Ramsay Method for Iron

SPECIAL REAGENTS

Concentrated Sulfuric, Perchloric (60%), and Nitric Acids. Test the nitric acid for iron and distill from glass, if necessary.

50% Potassium Thiocyanate.

0.02 to 0.05 N Titanous Sulfate (depending on the quantity of iron and the bore of the burette). Pour 200 ml. freshly boiled distilled water into bottle *F* (Fig. 93) with stopcocks *C*, *D*, and *G* closed. Mix the proper amount of com. 15% titanous sulfate or chloride with 10 ml. water and 10 ml. conc. sulfuric acid. Boil the mixture vigorously for 1 min. Pour at once into *F*; stopper immediately. Open stopcock *D* and let stand overnight for any precipitate to settle. Then close *D*, open *C*, and allow a brisk stream of hydrogen to bubble through *A*. Close *C*, and open first *D* and then *G* to force the standard soln. over the siphon into the burette. A soft vacuum grease (*W. Edwards and Co.*) was used for the stopcocks, but perhaps silicone grease would be better. Standardize daily against a standard ferric ammonium sulfate soln.

Standard Ferric Ammonium Sulfate. Prepare a soln. in 3 *N* sulfuric acid such that 5 ml. requires a vol. of titanous sulfate equivalent to 100–200 mm. on the burette.

Sodium Chloride (Powdered).

50% Formic Acid in Ether. Redistill each component from glass, and mix just before using.

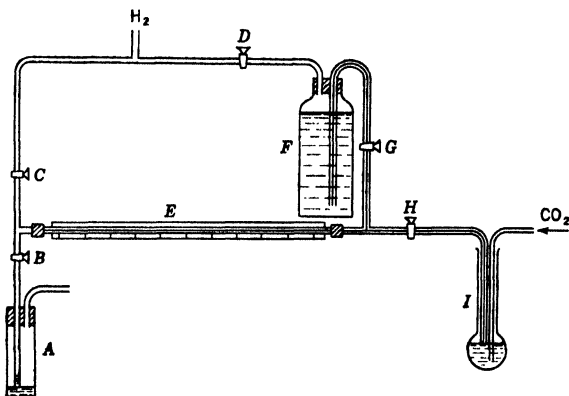



Fig. 93. Modified Conway burette. *A,* pressure-regulating vessel; *B, C, D, G,* and *H,* stopcocks; *E,* the burette, standard-bore capillary tubing against mm. scale (10 cm. ≈ 0.05 ml.); *F,* $Ti_2(SO_4)_3$ reservoir, capacity 200–250 ml.; *I,* digestion and titration flask. *From Ramsey (1944)*

PROCEDURE

1. Add the sample (0.2 ml. blood) to 0.2 ml. each of the nitric and perchloric acids and 0.5 ml. sulfuric acid in a digestion flask. Add a boiling chip and heat with shaking over a micro flame. The mixture darkens, then clears, and just before the last of the perchloric acid boils off the mixture becomes a clear deep greenish-yellow for 3–5 sec. Continue heating for 3 sec. after the disappearance of this color.

2. If copper is present, dilute the cooled digest with 2 ml. water and transfer as completely as possible to a narrow test tube. Rinse the flask with two portions of 1 ml. water and add the rinsings to the test tube. Add 0.5 ml. sulfuric acid and saturate the soln. with sodium chloride. Shake the tube twice with 2 ml., and twice with 1 ml. 5%

formic acid in ether. Transfer the ether extracts back to the digestion flask and completely evaporate the ether over warm water. Digest again after the addition of 0.5 ml. sulfuric acid and a drop or two each of the nitric and perchloric acids. Cool the clear digest and add 5 ml. water.

If too little copper is present to interfere, omit this step. (The separation of copper is not necessary for blood analysis.)

3. Add 1 ml. 50% potassium thiocyanate to the diluted digest, bubble carbon dioxide through the soln., open stopcock *H*, and deliver the titanous sulfate until the last trace of pink just disappears. To control the rate of delivery adjust the height of the liquid column in the tube leading from *A* to *B*, and carry out the last of the titration with *C* closed and *B* open.

PHOSPHORUS

A method for the measurement of phosphorus in the range of 0.5–10.0 μg. was given by Lindner and Kirk (1937b). The method consists of conversion of the phosphorus to phosphate by ashing, precipitation as ammonium phosphomolybdate, isolation of the precipitate by filtration through a sintered-glass filter stick with an asbestos mat, solution of the precipitate in a measured excess of sodium hydroxide, addition of an equivalent amount of acid, and finally titration of the excess acid with alkali to a phenolphthalein end point. The end point is not sharp and the precision of the method leaves much to be desired.

In an early review, Blick (1935a) reported that, with Linderstrøm-Lang and Holter, he had developed a method for inorganic phosphate capable of determining 5 μg. phosphorus with an error of less than 1%, and 1μg. with an error of about 5.5%. In this method the phosphate was precipitated as magnesium ammonium phosphate, the precipitate was centrifuged, washed with alcohol and then acetone, and dissolved in an excess of standard hydrochloric acid. The excess acid was titrated in an acetone medium with ammonium acetate using naphthyl red indicator. Phosphoric acid does not ionize in acetone; therefore only the free hydrochloric acid is titrated. The acetate ion is used like an alkali in this case since it will react with hydrogen ions to form acetic acid which, in acetone, does not ionize.

Both of these titrimetric methods lack the ease and simplicity

characterizing the colorimetric procedures (page 226), and, since the former methods do not offer sufficient compensating advantages in accuracy, in the opinion of the writer, the colorimetric methods deserve preference.

CHLORIDE

In chronological order, the following methods have appeared for the determination of chloride in very small amounts of biological material.

Linderstrøm-Lang, Palmer, and Holter (1935) described an electrometric titration method that can be carried out with a precision of 0.02 μl. 0.02 *N* silver nitrate. The sample for analysis should contain preferably 0.4–2.0 μg. chlorine. For use on tissue, a preliminary ashing procedure was employed which could be conducted in the titration tube itself to avoid transfer.

Conway (1935) employed a method based on oxidation of the chloride to chlorine by an acid permanganate reagent, absorption of the chlorine by potassium iodide solution, and estimation of the iodine liberated by either titration or colorimetry, depending on the quantity present. The reaction is made to take place in a diffusion cell. Conway reported that, for quantities of chloride corresponding to about 0.3 mg. chlorine, the coefficient of variation for a single determination is 0.5%. Down to 7 μg. chlorine the coefficient of variation increases to 4–5%, and when the method is adapted to 0.7 μg. the coefficient of variation becomes 6–7%.

Wigglesworth (1937) used a method based on addition of excess silver nitrate, filtration, and back-titration with thiocyanate. The method is applicable to as little as 0.3 μl. tissue fluid and the precision is ± 6%. Dean (1941) modified the Wigglesworth method and carried out the reaction and titration in a drop placed on a waxed slide.

Cunningham, Kirk, and Brooks (1941) described an electrometric titration method similar to that of Linderstrøm-Lang, Palmer, and Holter (1935). A shallow dish is employed as the titration vessel and silver-silver amalgam electrodes, a stirring needle, and the burette tip all dip into the solution. To determine the end point of the titration, the volume of standard silver nitrate solution added is plotted against the resulting E.M.F. values. A sample containing

0.5–30 μg. chloride is used. For more than 2 μg. the error does not exceed 0.5%, and for more than 0.5 μg. it does not exceed 2%.

Since there is little difference between the two electrometric methods, only that of Linderstrøm-Lang, Palmer, and Holter will be described. It has a simple electrode system (the filled burette serves as one half of the cell), and the titration can be carried out in the glass reaction tube used for the ashing of the sample. Cunningham *et al.* (1941) demonstrated that plasma can be titrated directly, without ashing, by the electrometric method. Colorimetric methods are given on pages 200 and 224.

Linderstrøm-Lang, Palmer, and Holter Electrometric Method for Chloride

SPECIAL APPARATUS

The arrangement of the titration set-up is illustrated in Figure 69. *A* and *A′* are silver wires that serve as electrodes. They are connected to a potentiometer so arranged that the galvanometer gives no deflection when the potential difference across the electrodes is +210 millivolts, corresponding to a silver concentration in the titration tube of 0.05×10^{-4} *M*. This concentration would be obtained by adding 0.013 μl. 0.02 *N* silver nitrate to the 50 μl. liquid employed in the titration tube. The burette, which is of the type 2 variety (Fig. 83, page 256) can be read to 0.02 μl.

SPECIAL REAGENTS

Ashing Solution. Dissolve 1.2 g. sodium nitrate and 0.4 g. secondary sodium phosphate ($Na_2HPO_4.2H_2O$) in water and make up to 14 ml.

0.10 N Nitric Acid. This soln. and the titration soln. below were changed from 0.17 to 0.10 *N* nitric acid, as suggested by Linderstrøm-Lang (1936) in a later publication.

Titration Solution. Dissolve 0.3398 g. silver nitrate (0.02 *N*) in 100 ml. 0.10 *N* nitric acid containing 1.20 g. sodium nitrate and 0.40 g. secondary sodium phosphate ($Na_2HPO_4.2H_2O$).

PROCEDURE

1. Place the tissue sample in a heat-resistant tube (Fig. 35, page 167) containing 7 μl. ashing soln. Evaporate the water in an oven at 106°, and ash the material by careful heating in a flame.

When a crucible oven is available, first heat to about 300° for 10 min. and then continue to heat over the flame. Employ the coolest flame that will burn away the brown tar which forms.

2. Add 50 μl. 0.10 *N* nitric acid with a hand pipette.

3. Titrate the liquid in the tube with the titration soln. keeping the galvanometer circuit closed and closing the titration circuit by bringing the liquid in contact with the burette tip. Observe the deflection each time the tip touches the liquid and a little of the titration soln. runs out. Close the titration circuit only momentarily to prevent polarization. When the deflection changes direction, the end point has been reached.

NITROGEN AND AMMONIA

A discussion of various methods for the titrimetric determination of total nitrogen and ammonia has been included in the section dealing with the colorimetric methods (page 233). Since the method developed at the Carlsberg Laboratory is the most precise (0.005 μg. nitrogen) and has had its worth established by the most critical trial over many years, the procedure finally recommended, and given by Brüel, Holter, Linderstrøm-Lang, and Rozīts (1946), will be the only one presented. In its present form the range of the method is 0.1–1 μg. nitrogen, but larger quantities may be determined by increasing the concentration of the reagents. The digestion of the sample by the method of these investigators has already been described on pages 234–236). To avoid the use of alkaline solutions in the microburette, standard acid is employed for the titration after an excess of dilute alkali has been added to the acid used to absorb the ammonia.

Brüel, Holter, Linderstrøm-Lang, and Rozīts Method for Nitrogen and Ammonia

SPECIAL MATERIALS

Paraffin Block. Smooth the surface of a block of paraffin by scraping with a knife. Store the block under distilled water, and dry with filter paper when removed from the water for use.

SPECIAL REAGENTS

18 N Sodium Hydroxide.

0.015 N Sulfuric Acid.

Alkaline Indicator Solution. Add 0.5 ml. $M/15$ secondary sodium phosphate to 2 ml. 40 milligram per cent bromocresol green (pH 4.6) and 2.5 ml. boiled water. Prepare fresh each day and keep in well-closed vessel.

0.01 N Hydrochloric Acid containing 10 ml. 40 milligram per cent bromocresol green/100 ml.

Color Standard. Prepare by titrating a blank (45 μl. water + 7 μl. 0.015 N sulfuric acid + 18 μl. indicator soln.) with the 0.01 N hydrochloric acid until the color matches that of the indicator in citrate buffer at pH 4.6. The slight difference in shade of the indicator in citrate buffer makes the titrated blank the better color standard.

PROCEDURE

1. Pipette a drop A of distilled water (about 20 μl.) onto a clean paraffin block (Fig. 94).

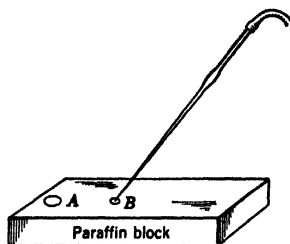



Fig. 94. Use of the paraffin block in transfer of the digest. *From Brüel et al. (1946)*

2. Draw up about one third of A into a hand pipette without a constriction (Fig. 55, page 175) and carefully introduce the stem of the pipette into the digestion tube until the tip touches the center of the concave bottom of the tube. See that the pipette stem is properly centered in the tube so that liquid is not drawn up along the walls. Warm the tube just before adding the water to prevent crystallization of potassium sulfate, which might block the pipette.

3. Blow out the water into the digested sample (prepared as described on pages 234–236) and immediately suck up the mixture into the pipette. Deposit the liquid as a drop (B) on the paraffin block.

4. Draw up another third of A into the pipette and use the water to wash the walls of the digestion tube. Draw the liquid back into

the pipette. The last of the liquid usually follows the tip of the pipette as it is withdrawn along the wall of the tube and can be sucked up near the rim. Add the liquid in the pipette to *B*.

5. Repeat the rinsing of the tube with the last third of drop *A*. With care, the last of *A* can be removed from the paraffin without drawing air through the pipette tip. Add the liquid to *B*.

6. Draw up drop *B* completely into the pipette and transfer it to the bottom of the paraffined distillation tube (page 169 and Fig. 35, page 167). It is essential that the pipette does not touch the side walls of the tube. Accordingly, clamp the pipette vertically and raise up the tube on a pipetting stand so that the centering of the pipette stem will be proper. Deposit the liquid in the bottom of the tube. It is usually permissible to ignore the film of liquid adhering to the walls of the pipette after it has been emptied.

7. Add 9 μl. 18 *N* sodium hydroxide directly to the liquid in the bottom of the paraffined tube with the same type of pipette used for the previous steps. Again it is essential to avoid contact between the pipette and the upper walls of the tube. Use the pipette stand for the operation as in the previous step. Immediately after addition of the alkali place a seal of 45 μl. water across the neck of the upper part of the vessel, using a constriction pipette of the type shown in Figure 58, as described on page 174.

8. Pipette 7 μl. 0.015 *N* sulfuric acid into the water seal at once using a constriction pipette (Fig. 57, page 175) which is emptied by constant air pressure adjusted so that the pipette will deliver when the tip touches the water. This arrangement insures greater accuracy.

9. Stopper the tube with a rubber cap plugged with a drawn-out piece of glass tubing, rather than a piece of glass rod, to prevent displacement of the seal (Fig. 46, page 171).

10. Immerse the bottom half of the tube in a thermostat at 40° for 1.5 hr.

11. Add 18 μl. alkaline indicator soln. to the receiving seal by means of a constriction pipette which is emptied by constant air pressure.

12. Titrate with the 0.01 *N* hydrochloric acid to the color of the color standard using a "flea" stirrer. About 1.5 μl. of the acid will be required to bring the blank without absorbed ammonia to the end point.

13. Run controls in which the complete analysis is duplicated without the unknown sample.

UREA

Urea has been determined by the urease method in a number of laboratories by making use of one or another of the micro procedures for the estimation of ammonia. In the urease method, the enzyme is added to decompose the urea, and the ammonia thus formed is absorbed in acid and measured.

Procedures employing diffusion cells were described by Conway (1933), Gibbs and Kirk (1934), Borsook (1935), and Kinsey and Robison (1946). The various forms of diffusion cell used by these investigators have been described (page 168). The method of Kinsey and Robison (1946), which can estimate as little as 1 μg. urea in 4 μl. plasma or aqueous humor with a precision of ±5%, will be described. The methods which do not utilize the diffusion cell for the estimation of ammonia can also be applied in the present case. Thus, the method of Linderstrøm-Lang and Holter (1933b) (page 230) was developed with the application to the measurement of urea in mind as one of the potentialities. A colorimetric method is given on page 214.

Kinsey and Robison Method for Urea

SPECIAL REAGENTS

Glycerol-Boric Acid. Add 25 ml. pure glycerol to 100 ml. 25% glycerol saturated with boric acid.

Urease Extract. Dissolve 1 g. powdered double strength urease (*Squibb*) in 100 ml. boiled saturated sodium chloride.

Saturated Sodium Metaborate in Saturated Potassium Chloride (boiled).

0.002 N Hydrochloric Acid.

Gramercy Universal Indicator (Fisher Scientific Co.) diluted with 15 parts distilled water.

Mineral Oil.

PROCEDURE

1. Measure the sample into the central container of the diffusion

cell (Fig. 43, page 168). Add 1 drop urease soln., stir with a "flea" or otherwise, and allow to react for 20 min. at room temperature.

2. Place a few drops of mineral oil in the well, and pipette 5 μl. glycerol-boric acid into the center of the covering vial.

3. When the digestion is completed, add 1 drop metaborate soln. to the reaction mixture, and immediately place the covering vial over the central container. Stir the digestion mixture and allow 60 min. at 30° for the diffusion.

4. Remove the covering vial, blot off the adhering mineral oil, add 0.5 ml. diluted indicator, and titrate with 0.002 *N* hydrochloric acid to the color of a control containing no urea. (The latter should be yellow; a greenish appearance indicates some ammonia has been introduced, probably from the sodium chloride used in the urease soln. or from the potassium chloride-metaborate soln.) Allow about 1 min. between the addition of the final increment of acid and the matching of the end point.

NOTE: The colorimetric procedure of Russell (1944) (page 238) may be substituted for the titration: Wash out the glycerol drop from the vial with three 0.5 ml. portions of water into colorimeter tubes. Add 0.05 ml. 0.003 *M* manganese sulfate or chloride to each tube. Chill the tubes and add 1.0 ml. alkaline phenol reagent (25% phenol in 2.7 *N* sodium hydroxide) and 0.5 ml. hypochlorite soln. Place the tubes in a boiling water bath for 5 min., cool, dilute to a convenient vol. and read in a colorimeter.

UREASE

Procedures for the determination of urea can, of course, be adapted to the measurement of urease. This was done by Linderstrøm-Lang and Holter (1940, page 1144), who based the method on their ammonia determination (page 230). 7 μl. enzyme soln. and 7 μl. substrate (1.2 g. urea, 50 ml. 0.15 *N* sodium hydroxide, and 50 ml. 0.3 *M* primary potassium phosphate, *p*H 6.8) were employed. The ammonia was liberated from the digested mixture by drawing into it 7 μl. 2 *N* sodium hydroxide, which had previously been placed on the side of the reaction tube. The distillation of the ammonia and the titration were then carried out (page 285).

The determination can also be carried out by measuring the ammonia in an acetone titration according to the procedure used by Linderstrøm-Lang and Søeberg-Ohlsen (1936). To the reaction

mixture given above, 150 μl. acetone containing naphthyl red is added, and titration with 0.05 *N* alcoholic hydrochloric acid is performed in the usual manner (page 303).

AMIDE, PEPTIDE, AND NITRATE NITROGEN

Borsook and Dubnoff (1939) described methods for amide, peptide, and nitrate nitrogen which were adapted to 0.1 ml. samples containing as little as 0.3 μg. nitrogen. These methods all involve determination of ammonia, a diffusion cell technique, and an electrometric titration, a glass electrode being used by the authors. Whether or not one wishes to follow this particular procedure for the estimation of ammonia, the initial steps in the methods of Borsook and Dubnoff can still be utilized; the details are given below.

Borsook and Dubnoff Methods for Amide, Peptide, and Nitrate Nitrogen

Amide Nitrogen

SPECIAL REAGENTS (in addition to those required for determination of ammonia)

3 N Sulfuric Acid.

2.9 N Sodium Hydroxide.

PROCEDURE

1. Pipette 0.2 ml. sample into a Kjeldahl digestion tube. Add 0.1 ml. 3 *N* sulfuric acid, and mix.
2. Cover with a tin-foil cap and place in a boiling water bath for 3 hr.
3. After the hydrolysis, cool and add 0.1 ml. 2.9 *N* sodium hydroxide, mixing well during the addition.
4. Determine the ammonia nitrogen and subtract from this value the ammonia nitrogen present before hydrolysis as measured by a separate analysis. The difference is the amide nitrogen.

Nitrate Nitrogen

SPECIAL REAGENTS (in addition to those required for determination of amide and ammonia nitrogen)

Devarda Alloy (finely powdered).

25% Sodium Hydroxide.

PROCEDURE

1. To the soln. remaining after all ammonia has been distilled out, in the determination of amide nitrogen, add 4 mg. Devarda alloy. Tilt the vessel so that the alloy is separated from the liquid.
2. Then introduce 0.1 ml. 25% sodium hydroxide, and allow the ammonia formed to diffuse into a fresh drop of standard acid. 1–2 hr. is sufficient for the reduction of the nitrate, and the distillation of the ammonia will be complete within this period if the diffusion cell of the authors is employed.
3. Determine the amount of ammonia which was absorbed.
4. Employ two sets of controls, one in which the sodium hydroxide is added without the alloy, and a second in which the alloy is added to a control mixture containing the reagents for the amide determination, but no sample. Substract the sum of these two controls from the titer obtained in step 3.

Peptide Nitrogen

SPECIAL REAGENTS (in addition to those required for measurement of amino acid and ammonia)

Peptidase Preparation (ammonia free). Seed skim milk containing 5% dextrose with spores of *Aspergillus wentii* and incubate for 6–8 days at 30°. Culture the mold in 1 l. Erlenmeyer flasks with a 2 cm. depth of medium. A satisfactory growth is obtained when the heavy pad of mold has begun to crack and draw away from the wall of the flask. Remove the pads; weigh, and freeze for several hr. to inactivate any arginase which may be present. Grind the pads in a mortar with sand and add enough water to give a suspension containing about 10% dry matter. (Determine the moisture on a small sample of the pad.) Bring the *p*H to 7 to 7.5 and cover the suspension with toluene. After standing 4–6 hr. at room temperature, filter through sailcloth with the aid of a vacuum. Add an equal vol. acetone to the filtrate, and dissolve the precipitate which forms in one fourth the original vol. water. Centrifuge, and shake the supernatant with permutite to remove ammonia. Store this enzyme soln. under toluene in a refrigerator. The activity will be maintained for months even though the soln. will darken on standing.

PROCEDURE

1. Add an equal vol. of a protein-free extract (trichloroacetic

acid filtrate), brought to *p*H 6.0, to the enzyme soln., and incubate overnight under toluene.

2. Determine the ammonia and carry out a formol titration. Multiply the ammonia found by 1.1 and subtract this value from the formol titration value at the second stage. From the figure obtained, subtract the sum of the formol titer of the enzyme soln. and the free amino nitrogen before the enzymatic hydrolysis to arrive at the peptide amino nitrogen.

ACID, ALKALI, AMINO, AND CARBOXYL GROUPS

Titration of acid or alkali in the conventional manner with micro apparatus, using indicators or electrometric means for determining the end point, requires little discussion. Usually it is advisable to use a color standard for matching the end point when indicators are employed. The use of standard acid in capillary burettes offers no difficulties; however, standard solutions of metallic hydroxides often give rise to the complications of carbonate precipitation in the fine lumen, and for this reason have been avoided by the Carlsberg Laboratory group. The difficulty has been circumvented in some cases by the use of tetramethylammonium hydroxide for the standard solution or by the addition of excess alkali and back-titration with acid. With alkaline end points, protection from the carbon dioxide of the air must be afforded. Methods for accomplishing this have been given on page 257. For electrometric titrations, various arrangements may be employed, such as the metallic wire electrode of Linderstrøm-Lang, Palmer, and Holter (1935) (Fig. 69, page 184) or the open-cup glass electrode of Sisco, Cunningham, and Kirk (1941) (Fig. 70).

For the direct microtitration of amino groups, Linderstrøm-Lang and Holter (1932) developed an acidimetric acetone titration method, which is described in their procedure for proteolytic enzymes (page 303). Linderstrøm-Lang and Duspiva (1936) employed an alkalimetric alcohol titration method for the direct micro estimation of carboxyl groups, and this measurement is described in their protease method (page 304). Glick (1934) (page 309) determined carboxyl groups by their neutralization in an excess of alkaline buffer and acidimetric titration to an acid *p*H. An indicator formol

titration was described by Weil (1936) (page 306), and an electrometric formol procedure by Borsook and Dubnoff (1939). Sisco, Cunningham, and Kirk (1941) employed their glass electrode (Fig. 70, page 184) for the electrometric titration, and they also used an indicator method in which the titration is carried out in an open drop but differing little from the procedure of Weil.

LIPID

A clever microtitration technique for the measurement of lipid in quantities of the order of 10 μg., suited for the determination on 1 mg. of tissue, with a precision of about 1% was developed by Schmidt-Nielsen (1942). The method is based on saponification of the lipid in a sealed capillary tube by alcoholic alkali in the presence of toluol, liberation of free fatty acid by the addition of an excess of mineral acid, solution of the fatty acid in the toluol phase, and titration of an aliquot of the toluol layer after the toluol has been evaporated.

Schmidt-Nielsen Method for Lipid

SPECIAL REAGENTS

Pure Toluol.
1 N Potassium Hydroxide in Absolute Alcohol (aldehyde free).
0.5 N Hydrochloric Acid.
0.01% Thymol Blue in Absolute Alcohol.
0.02 N Alcoholic Tetramethylammonium Hydroxide.

PROCEDURE

1. Pipette the lipid sample in 20 μl. toluol into the bottom of a thin-walled capillary tube having an internal diameter of about 1.8 mm. and a length of about 50 mm. Clamp the pipette vertically and raise the capillary tube, placed in a holder, on a mechanical stand so that the tip of the pipette will not touch the sides of the tube as it is raised. Use constriction pipettes for all pipettings. Exercise care to avoid evaporation of toluol in steps 1–6.
2. In the same way pipette 2.5 μl. alcoholic potassium hydroxide into the toluol soln. and immediately seal the opening of the capillary.

3. Set aside overnight at room temperature or heat to over 80° for 10 min. to effect saponification. Keep the material at the one end of the tube so that none will be lost when the other end is subsequently cut off to introduce the next reagent.

4. Centrifuge to drive down the droplets of toluol which condense on the upper walls of the capillary, open the empty end of the tube with a diamond point, and pipette an excess of 0.5 N hydrochloric acid down into the tube (13 μl. will suffice in most instances).

5. Reseal the open end of the capillary at once and thoroughly mix the contents by shaking. The special centrifuging apparatus which repeatedly turns the tubes (Fig. 63, page 178) has been especially designed for mixing liquids in capillary tubes.

6. Separate the toluol and water phases by centrifuging, open the capillary, and pipette an aliquot of about 13 μl. of the toluol layer into a titration vessel with a standard ground mouth (Fig. 86).

7. Evaporate the toluol in a desiccator furnished with paraffin chips at about 30 mm. mercury pressure.

8. Dissolve the fatty acid in 100 μl. 0.01% alcoholic thymol blue, add a stirring "flea," and titrate with the standard tetramethylammonium hydroxide using the set-up shown in Figure 86 to exclude atmospheric carbon dioxide. Match the color to that of a faint green color standard prepared by titrating 100 μl. alcoholic thymol blue to a pure yellow color and then adding 5 μl. 0.04% bromophenol blue, which has been rendered blue with a little sodium hydroxide.

9. Run a blank titration on 100 μl. alcoholic thymol blue.

EXTRACTION AND FRACTIONATION OF LIPIDS

To enable the extraction and fractionation of the lipids in quantities of tissue of the order of 1 mg., so that analytical methods might be applied to the material, Schmidt-Nielsen (1944b) elaborated appropriate procedures. The tissue lipids are treated with alkali and the unsaponifiable fraction is extracted with toluol. Fatty acids are liberated from the aqueous portion by the addition of mineral acid and then they are extracted with toluol.

Schmidt-Nielsen Method for Extraction and Fractionation of Lipids

SPECIAL REAGENTS

Pure Toluol.
1 N Potassium Hydroxide in Absolute Alcohol (aldehyde free).
4 N Hydrochloric Acid (chlorine free).

PROCEDURE

A. Unsaponifiable Fraction

1. Place the tissue in the bottom of a capillary tube (1.8–2 mm. internal diameter, 50 mm. long, and sealed at one end). Two microtome sections each 25 μ thick and 5 mm. in diameter weighing approximately 1 mg. may be used. It is convenient to employ the cryostat microtoming technique (page 427), in which the sections are allowed to curl up on the knife edge and are transferred into the capillary tube kept cold in the cryostat.
2. Pipette 2.5 μl. alcoholic potassium hydroxide into the tube with the sample and follow with the addition of 20 μl. toluol. Seal the open end of the tube at once. Follow pipetting directions on page 291.
3. Saponify by heating the tube to 80–100° for 2 min. Centrifuge to throw down droplets of toluol that condense along the tube.
4. Open the tube, introduce 16 μl. water, and reseal.
5. Mix the liquids thoroughly by placing in the mixing centrifuge (page 178).
6. Separate the emulsion into two layers by centrifuging at 3500 R.P.M. or faster, after heating the capillary tube, the ordinary centrifuge tube into which it is placed, and the metal centrifuge tube holder in an oven at 110°. If the separation is incomplete, reheat and recentrifuge.
7. Open the tube and pipette a 16 μl. aliquot of the toluol phase into a paraffined reaction tube (page 169). Evaporate off the toluol in a desiccator over paraffin shavings at a pressure of 20 mm. mercury.

B. Saponifiable Fraction

1. Evaporate the toluol left in the aqueous phase from step *A*7 in the desiccator, as above. Should the iodine number subsequently be determined on the saponifiable fraction it would be necessary to correct it for the presence of the remaining unsaponifiable lipid. This would require that the iodine number of the unsaponifiable fraction be determined also. It is impossible to obtain complete separation of the two phases by pipetting.
2. Add 2.5 μ 1.4 *N* hydrochloric acid and 20 μ toluol, and seal the capillary tube as before.
3. Mix as in step *A*5 and separate the layers by centrifuging without heating.
4. Remove an aliquot of the toluol phase as in step *A*7. If the sample is to be used for titration of fatty acids, transfer the aliquot to a titration vessel with the standard ground mouth (Fig. 86). If the iodine number is to be determined, transfer to a paraffined reaction tube. Evaporate the toluol as in step *A*7.

IODINE NUMBER OF LIPIDS

Schmidt-Nielsen (1944a) employed the principle of Kaufmann (1926) to develop a method having a precision of about 1% for the determination of the bromine-combining power of lipids in samples of the order of 10 μg. Unsaturated bonds in the lipid are saturated with bromine and the excess bromine is titrated iodometrically. The iodine number can be calculated from the titration value if the amount of lipid is also measured (page 291). Schmidt-Nielsen's innovation of using glycol monobutyl ether (butyl Cellosolve) for the fat solvent has the advantage that the low vapor pressure of the liquid obviates difficulties that would result in a micro method from evaporation. As the solvent is also miscible with water, it has the additional advantage of enabling the titration to be performed in a single liquid phase.

Kretchmer, Holman, and Burr (1946) employed a similar method for 10–100 μg. samples of lipid in chloroform solution, using 0.05 *N* pyridine sulfate dibromide in glacial acetic acid as the brominating agent. At least 20 min. was required for completion of the reaction.

Schmidt-Nielsen Method for Iodine Number

SPECIAL REAGENTS

Cellosolve (glycol monobutyl ether). Redistill and store in the cold.

1% Potassium Iodide Solution. Store in the dark.

0.1% Soluble Starch Solution. Stir up 0.1 g. in 10 ml. cold water and pour, with stirring, into 90 ml. water at 76°. Store at 0°.

$^1/_8$ or $^1/_6$ N Bromine Solution. (May be made weaker.) Saturate methyl alcohol with sodium bromide (about 13 g./100 ml.); filter and add 10% more methyl alcohol. Dissolve the bromine in this soln. Store in the dark. Transfer some into a bottle with a small opening to minimize the evaporation of alcohol while being used.

0.05 N Sodium Thiosulfate. Stabilize by adding 0.2 g. sodium carbonate per liter.

Pure Toluol.

PROCEDURE

1. Pipette the sample dissolved in toluol into the bottom of a paraffined reaction tube (page 169).

2. Evaporate the toluol in a desiccator over paraffin shavings at a pressure of 20 mm. mercury.

3. Introduce a stirring "flea" and pipette 5 μl. Cellosolve into the bottom of the tube.

4. Place a seal of the potassium iodide soln. (about 45–50 μl.) across the lumen of the tube about one third of the tube length above the bottom, and follow with a similar seal of the starch soln. about one third from the top. The paraffin coating prevents the seals from running. Place the seals by holding the tube horizontally. Introduce the tip of the pipette to the proper position and have it just touch the wall. Blow out the liquid while rotating the tube. Finally cap the tube.

5. Use the "flea" to dissolve the lipid in the Cellosolve. Some creeping of the lipid occurs during the evaporation of toluol and all this lipid must be incorporated in the Cellosolve. The paraffin coating is insoluble in Cellosolve.

6. Introduce 2.5 μl. bromine soln. into the bottom of the tube,

from a pipette fixed vertically, by raising the tube on a mechanical stand so that the tip and shaft of the pipette pass through the two seals without touching the sides of the tube at any point.

7. After 5 min. the bromination is complete; then use the "flea" to draw the potassium iodide seal down into the liquid in the bottom of the tube and mix them.

8. Raise the tube on a stand so that the tip of the burette passes through the starch seal and dips into the bottom liquid without touching the sides of the tube. Add thiosulfate from the burette until the yellow color just disappears. Remove the tube, and with a quick snap of the hand throw the starch seal down into the bottom liquid. Replace the tube in the titration position and continue the titration to the end point. Use a previously titrated sample as a colorless control to facilitate judgment of the end point.

9. Run blank titrations on the bromine without a lipid sample.

REDUCING SUGARS

The iodometric method for the estimation of reducing sugars was adapted by Linderstrøm-Lang and Holter (1933a) to the micro scale. Their procedure has a precision of about 0.06 μl. of 0.05 N thiosulfate, which corresponds to 0.25 μg. glucose. Holter and Doyle (1938) introduced a few alterations in concentration and volume of the reagents and employed a reaction tube with a narrow neck (Fig. 38, page 167), which enabled an increase in precision to 0.04 μl. of 0.02 N thiosulfate.

Heck, Brown, and Kirk (1937) adapted the cerimetric method to the determination of reducing sugars with an average accuracy of about $\pm 1\%$. The cerimetric method was also used by Levvy (1946) for the determination of glucuronic acid. For a colorimetric method see page 210.

Holter and Doyle Modification of Linderstrøm-Lang and Holter Iodometric Method for Reducing Sugars

SPECIAL REAGENTS

0.4 M Carbonate Buffer (pH 10.2).

0.1 M Iodine in Potassium Iodide. Store in a black bottle connected by a siphon arm to a 6 μl. automatic pipette (Fig. 60, page 175).

1.2 M Sulfuric Acid.

0.5% Soluble Starch.
0.02 N Sodium Thiosulfate.

PROCEDURE

1. Pipette 7 µl. of the 0–1.5% sugar soln. into the bottom of a paraffined reaction vessel (page 169).
2. Add a stirring "flea" and pipette 10 µl. carbonate buffer into the tube.
3. Mix the buffer and sugar soln. and add 6 µl. iodine soln. from the automatic pipette, whose tip is below the surface of the liquid in the tube to prevent evaporation of iodine.
4. Directly afterward pipette 10 µl. 1.2 *M* sulfuric acid into the tube to form a seal across the narrow neck of the tube, and then place another seal of about 10 µl. starch soln. above the acid seal (Fig. 38, page 167). These seals prevent loss of iodine.
5. Stir the reaction mixture and let stand for 20 min.
6. Cap the tube and centrifuge for 1 min. at about 1500 R.P.M. to collapse the seals and cause them to mix with the reaction liquid.
7. Titrate the iodine with 0.02 *N* thiosulfate, using a microburette in which mercury is not in contact with the thiosulfate.
8. Run a control in which water is substituted for sugar soln.

Heck, Brown, and Kirk Cerimetric Method for Reducing Sugars

SPECIAL REAGENTS

14% Sodium Carbonate. Prepare from anhydrous salt.
1.5% Potassium Ferricyanide.
10% (by vol.) Sulfuric Acid.
0.01 N Ceric Sulfate.
0.01 N Ferrous Ammonium Sulfate in 0.1 N Sulfuric Acid. Store as directed on page 275.
0.0025 M Phenanthroline Ferrous Sulfate. Prepare as described on page 275.

PROCEDURE

1. Measure soln. of sample containing 1–12 µg. glucose, or that quantity of other sugar with equivalent reducing power, into a tube (3 mm. inside diameter, 35 mm. long) calibrated at 200 µl.*

* It would be well to simplify this step as indicated on page 165.

2. Add about 40 μl. carbonate soln. and the same vol. ferricyanide soln.

3. Add water to the calibration mark* and place in boiling water for 5 min.

4. Transfer to a porcelain titration dish with a pipette and use successive rinsings with water to insure a quantitative transfer.*

5. Add 50 μl. 10% sulfuric acid and a little phenanthroline indicator.

6. Titrate with ceric sulfate to the first permanent grass-green color.

7. Run control by substituting water for the sugar soln. 1 μg. glucose requires 2.556 μl. 0.01 *N* ceric sulfate.

NOTE: For the deproteinization of blood, Heck *et al.* pipette sample into calibrated tube having a vol. about ten times that of the sample. The pipette is then rinsed into the tube,* a vol. of 10% copper sulfate ($CuSO_4.5H_2O$) equal to that of the sample is added, and while stirring a similar vol. of 10% sodium tungstate in 2% sodium carbonate is added slowly. The soln. is made up to the calibration mark with water,* stirred, centrifuged, and the supernatant is drawn off and used.

GLYCOGEN

Heatley (1935) described a micro determination of glycogen based on the isolation of glycogen from tissue, acid hydrolysis, and estimation of the reducing sugar formed by the method of Linderstrøm-Lang and Holter (1933a). The procedure of Heatley may be applied to amounts of tissue of the order of 1 mg. The maximum error is less than ±2 μg. glycogen corresponding to ±0.3 μl. 0.05 *N* thiosulfate. The loss of about 1.5% which occurs during the precipitation of the glycogen is approximately compensated by standardizing the reagents through a control estimation of a glycogen solution of known strength. Subsequently Heatley and Lindahl (1937) extended the method to include the separation of desmo- and lyoglycogen, according to the principles given by Willstätter and Rohdewald (1934), and the measurement of the two forms individually. For a colorimetric method see page 247.

* It would be well to simplify these steps as indicated on page 165.

Heatley Method for Total Glycogen

SPECIAL REAGENTS

All of the reagents used for the determination of reducing sugars (page 296) are required, in addition to:

30% Potassium Hydroxide.
Absolute Alcohol.
0.6 N Hydrochloric Acid.
0.1 N Hydrochloric Acid.
0.05% Thymol Blue.
1 N Sodium Hydroxide.
Paraffin.

PROCEDURE

1. Place the tissue sample, which may be fixed in absolute alcohol to stop enzyme action, in about 35 μl. 30% potassium hydroxide in a reaction tube.
2. Cap the tube and heat in a steam bath for 20 min.
3. With the pipette used for the alkali, add 35 μl. water and then 105 μl. absolute alcohol.
4. Mix well with the aid of a platinum wire.
5. Immerse the tube in a steam bath for only a few sec. to flocculate the glycogen without having the contents boil out.
6. Centrifuge at 3500 R.P.M. for 15 min. and pipette off and discard the clear supernatant.
7. Wash the precipitate with about 100 μl. absolute alcohol; centrifuge and again discard the supernatant.
8. Repeat the washing process once more and remove the last traces of alcohol by placing the tube in a steam bath for a few min.
9. With about 35 μl. 0.6 *N* hydrochloric acid wash down the deposit on the upper walls of the tube.
10. Add a 20–30 mg. piece of paraffin and place the tube in the steam bath for 2.5 hr.
11. While the tube is still hot, invert and rotate it to spread an even film of paraffin over the upper walls of the tube.
12. Add a "flea" and use it to break through the thin film of paraffin on the surface of the liquid.

13. Add a little thymol blue soln. and bring to a bluish-grey color with 1 *N* sodium hydroxide. If the end point is passed, bring to the proper color with 0.1 *N* hydrochloric acid.

14. Set up a control by neutralizing 35 µl. 0.6 *N* hydrochloric acid to thymol blue.

15. Determine the glucose by the method of Linderstrøm-Lang and Holter (page 269).

Heatley and Lindahl Method for Desmo- and Lyoglycogen

SPECIAL REAGENTS

The same as in the preceding method for total glycogen.

PROCEDURE

1. Place the tissue in a little distilled water in a tared reaction tube cooled by ice.

2. Plunge the tube into a current of steam, and shake it at intervals for 10–15 min. while in the steam. A mechanical shaker is recommended.

3. Centrifuge, and transfer the supernatant to another tube.

4. Add another portion of distilled water to the residue and again heat and shake as before.

5. Centrifuge, and add the supernatant to that previously drawn off.

6. Make one or two more extractions in the same way and evaporate the water from both tubes. Dry the tubes at 100° for 2 hr. and reweigh. The sum of the increases in weight of the tubes represents the dry weight of the tissue sample taken. The extracted material contains the desmoglycogen and the material in the supernatant is the lyoglycogen.

7. Add 35 µl. 30% potassium hydroxide to the material in each tube and proceed with the glycogen determination as previously described.

ASCORBIC ACID

The method introduced by Tillmans and associates for the determination of ascorbic acid by titration with 2,6-dichlorophenolindophenol was applied by Glick (1935b) to measurements on microtome sections of tissue. This micro method has a reproducibility of ±0.1 µg. ascorbic acid. Since the publication of the procedure, the use of

metaphosphoric acid has replaced other acids for extraction because of the greater stability of the vitamin in this medium. Hence, in the procedure to be given, metaphosphoric acid rather than the acetic acid originally employed will be indicated. Ponting (1943) found that oxalic acid may also be used. Ascorbic acid can be titrated with the dye, and dehydroascorbic acid cannot be measured unless it is reduced to ascorbic acid by an agent such as hydrogen sulfide. This reduction is made preferably in a 3% metaphosphoric acid extract buffered to *p*H 3.5 to 3.7 with citrate (Bessey, 1938). In macro work the hydrogen sulfide is passed through the solution for about 15 min., and after standing at room temperature for 2 hr., the sulfide is removed by passing wet nitrogen through the liquid for 45–60 min. An application of the procedure to micro work would require the experimental determination of the proper time interval for each of these steps. For the colorimetric determination, see page 245.

Glick Method for Ascorbic Acid

SPECIAL REAGENTS

Saturated Sodium 2,6-Dichlorophenolindophenol Solution (sodium 2,6-dichlorobenzenoneindophenol). Shake a few mg. with 100 ml. warm distilled water. Cool, filter, and store in a refrigerator. The soln. may be used for about 10 days. [A stable soln. prepared in dioxane was described by Stone (1940): Distill the dioxane, discarding the first and last 10%. Stir a weighed quantity of the dye into the dioxane (about 0.4% equals 0.01 *M*) and add glacial acetic acid to 1% of the volume. Stir well for about 15 min., and filter through dry No. 42 Whatman filter paper. During titration the dioxane soln. used should not exceed 10% of the total volume.]

2–3% Metaphosphoric Acid. Store in a refrigerator and prepare fresh every few days.

Color Standard. Prepare a rose Bengal soln. (about 1 part per million) having a pink tinge when placed in a reaction tube and compared with a similar vessel containing water.

Standard Ascorbic Acid Solution. Prepare a fresh 0.1% soln. of crystalline L-ascorbic acid in 2–3% metaphosphoric acid.

PROCEDURE

1. Standardize the dye soln. against freshly prepared ascorbic acid soln. under the titration conditions employed for the unknown.

2. Place 50 μl. of the metaphosphoric acid soln. in a 250 μl. tube and add the sample (care must be exercised to prevent oxidation of the material prior to extraction with the acid). For work on tissue sections, the practice has been to freeze the tissue solid immediately upon removal from the source, and fresh-frozen sections (20–40 μ thick, 4–5 mm. diameter) are prepared and transferred individually as they are cut to separate tubes containing the acid extractant. If titration cannot be made at once, as in the case of serial-section titrations, the tubes may be kept in a freezing mixture until time for titration.

3. Titrate with the standardized dye soln. using a microburette in which the mercury does not come into contact with the dye soln. The end point is reached when the color of the unknown persisting for 5 sec. matches that of the color standard placed beside it. Employ active stirring throughout the titration.

AMYLASE

The Linderstrøm-Lang and Holter (1933a) method (page 296) for the determination of reducing sugars was used by Linderstrøm-Lang and Engel (1937) for the measurement of amylase activity in tissue sections of barley. Holter and Doyle (1938) employed the method for studies of amylase in protozoa.

In the barley work the enzyme was extracted with *M*/15 phosphate buffer (*p*H 5.3) and 7–8 μl. extract was added to a similar volume of substrate solution consisting of 1.5% soluble starch in phosphate buffer of the same *p*H. After an appropriate digestion period at 40°, the increase in reducing sugar was estimated. In much the same manner the protozoan enzyme was measured. The phosphate buffer (*p*H 6.0) extract was added to a 2% starch substrate containing 2% sodium chloride.

The relative proportions of enzyme extract and substrate solution, the length of the digestion period, and the *p*H of the buffer must be dictated by the requirements of the particular case.

PROTEOLYTIC ENZYMES

Titrimetric methods for the determination of proteolytic enzymes in small samples of biological material, such as microtome sections

of tissue, have been developed by the Carlsberg Laboratory investigators. Linderstrøm-Lang and Holter (1932) described a method for dipeptidase based on the acetone titration of amino groups. With DL-alanylglycine as the substrate, the procedure has a precision of about 0.08 µl. of 0.05 N hydrochloric acid corresponding to 5.6×10^{-5} mg. amino nitrogen. Holter and Linderstrøm-Lang (1932) extended the method to include proteases of both the peptic and catheptic type. The maximum deviation in this case amounts to about 0.16 µl. 0.05 N acid or 1.2×10^{-4} mg. amino nitrogen. By substitution of the appropriate substrate, the acetone titration micro method has also been used for aminopolypeptidase, carboxypolypeptidase and prolinepeptidase by Søeborg-Ohlsen (1941) and others. Buffers need not be added in these measurements, since the substrates have sufficient buffer capacity of their own.

The alcohol titration for carboxyl groups was adapted by Linderstrøm-Lang and Duspiva (1936) for the alkalimetric measurement of protease; their method has a precision of about 0.3 µl. 0.05 N alkali. Because of precipitation, solutions more acid than pH 8 cannot be titrated by this procedure.

Weil (1936) described a formol titration for the determination of tryptic activity on a similar scale, which he reported to be reproducible to ±0.1 µl. 0.05 N alkali.

The dilatometric method for peptidase is described on page 417.

Linderstrøm-Lang and Holter Acidimetric Acetone Method for Proteolytic Enzymes

SPECIAL REAGENTS

Enzyme Extraction Medium. 30 ml. 88% glycerol + 5 ml. $M/15$ primary potassium phosphate + 5 ml. $M/15$ secondary sodium phosphate made up with water to 100 ml.

Acetone Containing 2 Milligram Per Cent Naphthyl Red.

0.05 N 90% Alcoholic Hydrochloric Acid.

Substrates. The peptidase substrates are 0.2 M solns. of the racemic peptides containing sodium hydroxide to give the desired pH, *e.g.,* 0.036 M alkali gives a pH of 7.4 to 0.2 M alanylglycine. An exception is leucylglycine which must be used in 0.18 M soln. because of its sparing solubility.

Dipeptidase. Alanylglycine or leucylglycine.

Aminopolypeptidase. Alanylglycylglycine or leucylglycylglycylglycine; also glycylglycyl-D-alanine (Levy and Palmer, 1943).
Carboxypeptidase. Benzoylleucylglycine.
Prolinepeptidase. Glycyl-L-proline.
Cathepsin. 4% edestin in 0.008 *N* hydrochloric acid, *p*H 4.4.
Pepsin. 4% edestin in 0.056 *N* hydrochloric acid, *p*H 2.1.

PROCEDURE

1. Pipette 7µl. enzyme extraction medium into a 0.25 ml. reaction tube. Place tissue section in the liquid, and introduce a mixing "flea."
2. After standing for 1–2 hr. at room temperature for enzyme extraction, 7 µl. substrate soln. is pipetted in. The liquid is then mixed.
3. Cap the tube and suspend in thermostat for the chosen digestion period.
4. Set up a control experiment by carefully placing the substrate soln. on the side of the tube as a separate drop not touching the enzyme in the bottom. Since there is a tendency for the side drop to run down into the bottom drop, hold the tube in a horizontal position, cap, and suspend in the thermostat in a horizontal position.
5. Stop the reaction, after removing from the thermostat, by pipetting in 30 µl 0.05 *N* alcoholic acid and mixing. The automatic pipette shown in Figure 60 (page 175) is especially useful in this step.
6. Add 150 µl. acetone-naphthyl red soln. and titrate the yellow liquid with 0.05 *N* alcoholic hydrochloric acid to an orange end point. Place 200 µl. of a standard orange soln. in a reaction tube and mount this beside the titration tube so that the end points may be brought to the same color tone.

Linderstrøm-Lang and Duspiva Alkalimetric Alcohol Method for Proteolytic Enzymes

SPECIAL REAGENTS

Enzyme Extraction Medium. Glycerol-phosphate soln. prepared as for preceding method.
Absolute Alcohol Containing 0.05% Thymol Blue.
0.05 N 90% Alcoholic Tetramethylammonium Hydroxide.

End Point Color Standard. Mix methyl green, fuchsin, and picric acid to obtain the bluish-green color obtained at the titration end point.

Substrate. (*a*) Dissolve 12 g. casein in 8 ml. 1 *N* ammonium hydroxide in the presence of 0.05 ml. octyl alcohol and make up to 100 ml. with water. (*b*) Prepare buffer soln. by mixing 2 *N* ammonium hydroxide with 2 *N* ammonium chloride in the ratio *a/b* given in the following table. (*c*) Prepare a buffer-*p*H correction soln. for the casein by mixing 1 ml. of the chosen buffer soln. with the corresponding number of milliliters of alkali as indicated in Table VII and make up to 10 ml. with water. The sodium hydroxide may be used instead of the ammonium hydroxide to avoid high concentrations of ammonia. (*d*) Before the experiment mix 1 ml. of the 12% casein soln. with 1 ml. of the buffer-*p*H correction soln.

TABLE VII
Composition of Ammonia Buffers for Various *p*H Values

*p*H		*a/b*	Alkali, ml.		
18°	40°		2 *N* NH_4OH	8 *N* NH_4OH	2 *N* NaOH
8.58	8.0	1/8	0.22	—	0.20
9.19	8.7	1/2	0.48	—	0.32
9.80	9.3	2/1	1.56	—	0.52
10.10	9.6	4/1	3.00	—	0.60
10.40	9.9	8/1	5.96	—	0.66
11.00	10.5	52/1	—	6.17	0.76

PROCEDURE

1–4. These steps are the same as in the preceding method. However, since the absorption of carbon dioxide by the alkaline reaction mixture must be avoided, the tubes are capped with soda lime stoppers. (Fig. 47, page 171).

5. Stop the reaction by pipetting in 130 μl. alcohol-thymol blue soln.

6. Titrate to the bluish-green end point with the 0.05 *N* tetramethylammonium hydroxide, matching the color to the color standard. Protect from carbon dioxide during the titration by using the paraffin-oiled glass bead arrangement (Fig. 85, page 257), or the soda lime "desiccator" (Fig. 86, page 258).

Weil Formol Method for Tryptic Activity

SPECIAL REAGENTS

Enterokinase Solution. Prepared according to Waldschmidt-Leitz, (1924). Dehydrate and defat scrapings of hog duodenum mucosa with acetone, acetone-ether, and ether in this order. Grind and sieve the dried material, and extract it at 30° for 2 hr. with 50 ml. 0.04 *N* ammonium hydroxide per gram solid. Centrifuge, and concentrate the supernatant by evaporation at about 35°.

Veronal Buffer (*p*H 8.4). Add 8.23 ml. *M* sodium diethylbarbiturate to 1.77 ml. 0.1 *N* hydrochloric acid.

Substrate Solution. Bring 4% casein (Hammarsten) to *p*H 8.4 with 1 *N* sodium hydroxide.

Formol Solution. Neutralize 4 ml. 40% formaldehyde, to which 2 ml. 0.1% alcoholic phenolphthalein is added, with 0.1 *N* sodium hydroxide to the first pink color, and make up to 25 ml. with distilled water. Prepare fresh before use.

0.05 N Tetramethylammonium Hydroxide.

PROCEDURE

1. Place tissue section or enzyme preparation in 7 μl. enterokinase soln. in a reaction tube and let stand for 1 hr. at room temperature.
2. Add 7 μl. Veronal buffer and 7 μl. substrate soln.
3. Close the vessel with a soda lime tube (Fig. 47, page 171) and place in thermostat for the required digestion period.
4. Set up a control in which the buffer and substrate are placed on the side of the tube as a drop not touching the enzyme drop in the bottom of the tube. Place horizontally in thermostat to prevent the drops from touching.
5. Add 1000 μl. formol soln. and titrate with 0.05 *N* tetramethylammonium hydroxide to a marked red color matching against a standard. Protect from carbon dioxide in the air during the titration by one of the arrangements shown in Figs. 85 and 86, (pp. 257, 258).

ARGINASE

Two methods for the micro estimation of arginase were reported by Linderstrøm-Lang, Weil, and Holter (1935). The hydrolysis of the very strong base arginine yields the fairly strong base ornithine

and the very weak base urea. By titration to a *p*H at which the guanido group of arginine is completely ionized and the ε-amino group of ornithine (and urea) is not ionized, the cleavage of arginine can be followed as the increase in the quantity of base required to bring the reaction mixture to this *p*H. The increase in the amount of base will be one equivalent for each mole arginine hydrolyzed. This principle was employed by Linderstrøm-Lang *et al.* The proper *p*H was obtained by titration in acetone-alcohol with thymol blue indicator. This method has the advantage of convenience and rapidity; however, as the authors pointed out, it cannot be used if a large amount of urease is present because the subsequent action of the enzyme on the urea will increase the titration over the theoretical value of one equivalent per mole arginine. The urease activity is relatively low at the *p*H optimum of arginase, but the factor becomes appreciable nevertheless if high concentrations of urease are present. The titration of the ornithine set free is only about 95% of the theoretical, but this can be corrected if necessary by applying the factor 1.05 to the results.

The other method reported is based on the measurement of the ammonia formed when urease is allowed to act on urea set free by the action of the arginase. One volume of 0.10 *N* base in this method corresponds to one volume of 0.05 *N* base in the acetone-alcohol titration method.

Linderstrøm-Lang, Weil, and Holter Methods for Arginase

A. Urease Method

SPECIAL REAGENTS

Substrate Solution. (0.1 *M* arginine, *p*H 9.5). Dissolve 0.2104 g. arginine hydrochloride in 1.335 ml. 0.5 *N* sodium hydroxide and water to make a final vol. of 10 ml.

Urease-Buffer Solution. Prepare 100 ml. of a soln. containing 7 g. urease (*Squibb*) and 35 ml. 0.5 *M* phosphate buffer, pH 6.8 (mix equal vol. primary and secondary phosphate).

40% Sodium Hydroxide.

0.3 N Hydrochloric Acid containing 10 ml. 0.04% bromocresol purple/50 ml.

0.1 N Sodium Borate.

PROCEDURE

1. Pipette 7 μl. enzyme soln. and 7 μl. substrate into the bottom of the reaction tube, add a "flea," and mix. Close the tube with a rubber stopper and allow the reaction to proceed for a suitable time at an appropriate temperature. (Note that the type of tube originally used, Fig. 40, page 167, and the accompanying technique have been replaced by the method of Brüel *et al.*, 1946.)
2. Place the vessel in boiling water for about 15 min., cool, add 20 μl. urease-buffer soln., and stir with the "flea."
3. After allowing the vessel to stand 1 hr. at 20°, measure the ammonia formed using the method of Brüel *et al.* (1946) (page 283).

B. Acetone-Alcohol Titration Method

SPECIAL REAGENTS

Substrate Solution. Same as for urease method.

Acetone-Alcohol-Indicator Mixture. Prepare 100 ml. by adding a mixture of equal vol. acetone and absolute alcohol to 5 ml. of 0.1% alcoholic thymol blue.

0.05 N Tetramethylammonium Hydroxide in 90% alcohol.

End Point Color Standard. See page 292, step 8.

PROCEDURE

1. Pipette 7 μl. enzyme soln. and 7 μl. substrate into the bottom of a simple 250 μl. reaction tube (Fig. 35, page 167). Add a "flea," stir, stopper with a soda lime tube (Fig. 47, page 171), and allow the reaction to proceed at an appropriate temperature for a suitable period.
2. Stop the reaction by adding 150 μl. acetone-alcohol-indicator mixture.
3. Titrate with the tetramethylammonium hydroxide to the shade of the greenish end point color standard. Use one of the devices to exclude carbon dioxide during the titration (Figs. 85, 86, pp. 257, 258).

ESTERASES AND LIPASES

An acidimetric micro method for esterase was described by Glick (1934); this method was later applied to lipase by changing the substrate (Glick and Biskind, 1935). The method is based on the con-

tinuous neutralization of the acid liberated by an alkaline buffer medium. The loss in alkalinity of the buffer is then measured by titration with acid to *p*H 6.5, the magnitude of the titration being inversely proportional to the amount of substrate hydrolyzed. The greatest deviation in the determination is equivalent to about 0.14 μl. 0.05 *N* acid.

An adaptation was also made for the determination of cholinesterase (Glick, 1938). The sensitivity of this method is equivalent to the hydrolysis of 1×10^{-8} mole of ester. The titration is carried to an end point of *p*H 6.2. Sawyer (1943) reported a sharper end point and an increase in sensitivity when bromocresol purple is substituted for bromothymol blue and the end point is taken at *p*H 5.9. For a gasometric method see page 393.

Glick Acidimetric Method for Esterase and Lipase

SPECIAL REAGENTS

Enzyme Extraction Medium. 30% glycerol.

Buffered Substrates. *Esterase*: 1% methyl butyrate in a buffer soln. 0.1 *N* to sodium hydroxide and 0.4 *N* to glycine, *p*H 8.7 at 40°; prepare fresh before use. *Lipase*: Shake thoroughly a drop of tributyrin in a few ml. of the buffer soln. above; let stand for about 1 hr. in a refrigerator to allow the larger droplets to settle and use the supernatant homogeneous emulsion, or filter through paper and use the filtrate; prepare fresh before use.

Phenol-Indicator Solution. 10 ml. 2% phenol + 1.5 ml. 0.04% bromothymol blue.

0.05 N Hydrochloric Acid.

End Point Color Standard (*p*H 6.5). 3.1 ml. *M*/15 secondary sodium phosphate + 6.9 ml. *M*/15 primary potassium phosphate + 1 ml. 0.04% bromothymol blue.

PROCEDURE

1–4. These steps are carried out in the same fashion as the corresponding ones in the procedure for proteolytic enzymes (page 304).

5. Stop the reaction by adding 50 μl. phenol-indicator soln. with a constriction pipette.

6. Titrate with 0.05 *N* hydrochloric acid, matching the greenish color to that of the end point color standard. Take a uniform time

for each titration since a gradual bluing of the color occurs when the soln. stands at a *p*H near neutrality.

Glick Acidimetric Method for Cholinesterase

SPECIAL REAGENTS

Enzyme Extraction Medium. 30% glycerol.

Buffered Substrate Solution. 0.4% acetylcholine chloride in veronal buffer, *p*H 8.0 (7.15 ml. 0.1 *M* sodium diethylbarbiturate + 2.85 ml. 0.1 *M* hydrochloric acid.) Dissolve the substrate in the buffer just before use.

Eserine-Indicator Solution. Add 10 ml. 0.1% eserine sulfate to 1.5 ml. 0.04% bromothymol blue, or, according to Sawyer (1943), to 1.5 ml. 0.04% bromocresol purple.

0.05 N Hydrochloric Acid.

End Point Color Standard (*p*H 6.2 or 5.9). 10 ml. *M*/15 phosphate buffer + 1 ml. 0.04% bromothymol blue or bromocresol purple.

PROCEDURE

Identical with that in the preceding method with the substitution of the special reagents required for this enzyme.

CATALASE

Holter and Linderstrøm-Lang (1936) and Holter and Doyle (1938) described an adaptation to the micro level of the iodometric catalase method of Stern (1932). The estimation of the decomposition of hydrogen peroxide by the enzyme is made by thiosulfate titration of the iodine formed by oxidation of iodide placed in the reaction mixture. The precision of the titration is 0.06 μl. 0.02 *N* thiosulfate, which is equivalent to the decomposition of 0.02 μg. hydrogen peroxide.

Holter and Doyle Method for Catalase

SPECIAL APPARATUS

A 15 μl. constriction pipette for the substrate is surrounded by a water jacket, as shown in Figure 54 (page 173). Circulate ice water through the jacket to keep the soln. cold during the pipetting.

SPECIAL REAGENTS

Substrate Solution. 0.01 *M* hydrogen peroxide in 0.03 *N* phosphate buffer, *p*H 7.0. Prepare and keep at 0°.

Molybdic-Sulfuric Acid Solution. Make up 10 ml. saturated molybdic acid to 100 ml. with 33% sulfuric acid.

2% Potassium Iodide.

0.2% Soluble Starch in 0.5% Potassium Iodide.

0.02 N Sodium Thiosulfate.

PROCEDURE

1. Pipette 5–7 μl. enzyme soln. into a reaction tube (Fig. 38, page 167) coated with ceresine (page 169). Add a mixing "flea," and cool to 0°.

2. Add 15 μl. substrate soln. from the cooled pipette. Mix, and keep the liquid at 0°.

3. After a 1–2 hr. reaction period at 0°, add 10 μl. molybdic-sulfuric acid soln. and 10 μl. 2% potassium iodide.

4. Immediately after adding the iodide, place a seal of 20 μl. starch soln. across the lumen of the tube about 5 mm. above the surface of the reaction mixture to prevent loss of iodine.

5. After 3 min., pass the tip of the microburette through the starch film into the liquid below, and titrate with 0.02 *N* thiosulfate. After most of the thiosulfate has been added, draw the starch film down into the mixture and finish the titration.

IV. GASOMETRIC TECHNIQUES

As in the usual macro techniques, both volumetric and manometric methods have been employed for studies on single cells and small cellular aggregates of a well-defined nature. While some are simply refinements of the more macro methods which have been modified for the use of small quantities, other methods involve principles hitherto not applied to gasometric measurements, *e.g.*, Cartesian diver manometry.

No mention will be made of the Warburg or Barcroft apparatus not only because they are adapted to measurements of a relatively macro order, but also because they have been thoroughly treated in previously published works such as those of Dixon (1943) and Umbreit *et al.* (1945).

A. VOLUMETRIC

The gasometric techniques of histo- and cytochemical interest which have been based on volumetry have been developed chiefly for respiration studies. In addition to these, gas analysis techniques devised for use with very small volumes of blood will also be included, since they may prove useful in some investigations.

The advantage of volumetric over manometric apparatus for gasometric measurements depends largely on the fact that the former enables direct measurement of gas volume without the necessity of determining the volume of the apparatus. Furthermore, volumetric apparatus permits experimentation at constant pressure, which may be advantageous when a large proportion of liquid is present. However, the determination of the volume of gasometric apparatus is no great problem, and the choice of the technique need not be based on this factor. The particular nature and magnitude of the gas changes to be considered, combined with the availability of the apparatus and the personal tastes of the experimenter, will be more cogent factors in the selection of a technique.

1. Capillary Respirometry

The technique of volumetric capillary respirometry is based on the measurement of changes in the gas volume in a capillary tube connected to a chamber in which the respiring sample has been placed. The position of an index droplet placed across the lumen of the capillary serves as an indication of the volume at any given moment.

The history of the use and development of capillary respirometers has been documented in a review by Tobias (1943) and need not be presented here. In the following a description will be given only of a few of the more modern designs adapted to measurements on the histo- or cytochemical level.

The differential instruments consist essentially of two chambers connected by a capillary tube containing an index droplet. One chamber contains the biological material, medium, and reagents, and the other only the medium and reagents. Changes in the amount of gas which result from the biological action are followed by measurements of the displacement of the index droplet. In the closed system used, barometric fluctuations are without effect. When both chambers, drilled in a block of heat-conducting material, are of nearly the same size, temperature variations are of significance only in so far as they influence the rate of the reactions measured, and changes occasioned by the medium alone are cancelled. However, in this case, the displacement of the index droplet is only about half that obtained if the end of the capillary were open to the air. By employing a compensating chamber which is very large with respect to the reaction chamber, the movement of the index droplet in the differential instrument can be made to approximate that in the open type; but more careful temperature control will be required, since a given variation in temperature will take longer to exert its full effect on the larger chamber.

(*a*) *Cunningham-Barth-Kirk Differential Respirometer*

Cunningham and Kirk (1940) described a differential respirometer with chambers of approximately equal size, which has the advantages of enabling substitution of different capillary tubes, alteration of chamber volume, filling of the chambers with various gas mixtures as desired, and mixing of solutions during an experiment by means of an electromagnetic "flea" (page 179). Since the symmetrical

chambers are bored in a solid brass block, uniform temperature distribution is insured, and thermostasis is required only to control the rate of the reaction to be measured. The apparatus is adapted to

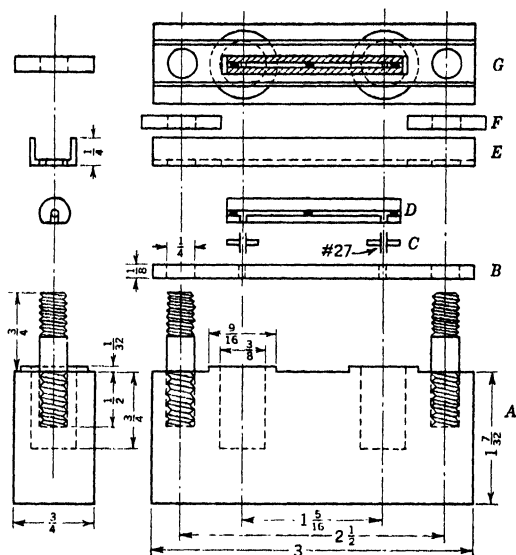



Fig. 95. Construction diagram of differential microrespirometer. Lengths are given in inches. *From Barth and Kirk (1942)*

measuring gas changes of the order of 0.1–10 μl. per hour with a reading accuracy down to 0.001 μl., depending on the capillary diameter. In an experiment on the respiration of single pupae of *Drosophila melanogaster* the mean deviation from the mean of the six organisms whose oxygen consumption was measured was 4×10^{-4} μl. per minute or an average of 2.4%. Barth and Kirk (1942) subsequently simplified the construction of the apparatus and rendered it more adaptable to multiple determinations; it is this apparatus which will be described (available from *Microchemical Specialties Co.*)

Apparatus. A diagram of the Barth-Kirk respirometer is given in Figure 95: The main block (*A*) is made from a 3 in. length of rectangular brass ($1\frac{1}{4} \times \frac{3}{4}$ in.). On one narrow face two chambers

are drilled symmetrically 1$^5/_{16}$ in. apart and $^3/_8$ in. diameter, and a machined rim is made around each chamber. Threaded bolts are placed at each end of the block as shown and a flat head plate (*B*) of Lucite (⅛ in. thick) is drilled to fit over the bolts. The head plate has the same dimensions as the top of the block and has a 1 mm. hole drilled over the center of each chamber. A glass capillary tube (*D*) with a bore 0.1–1.0 mm. and a length of 1¾ in. is flattened on one side by grinding to a width of ⅛ to $^5/_{16}$ in. and the ground surface is polished on a smooth stone to remove the frosting. Two holes separated by 1$^{15}/_{16}$ in. are drilled through the flat side into the capillary lumen; a blunt 27 gauge hypodermic needle may be used to drill the holes. Rubber gaskets (*C*), perforated by a short length of 27 gauge needle, are used to seal the capillary tube to the head plate. The capillary is cleaned with chromic acid mixture and the ends are plugged with paraffin and sealed by warming. A channel brass strip (*E*) is drilled to fit over the bolts so that by means of thin knurled nuts (*F*) it may be used to hold the capillary tube, head plate, and chamber block tightly together. The bottom of the channel brass is slotted over the entire length of the capillary to press only on the sides of the latter. The screws have to be slowly and evenly tightened to prevent the index droplet from being forced into one of the vertical connecting holes. All the surfaces to be sealed are coated with stopcock grease.

A paper scale may be placed under the center part of the capillary to enable reading of the displacement of the index droplet, or a low-power microscope fitted with an ocular micrometer may be used. The index droplet consists of kerosene which has been treated for several days with concentrated sulfuric acid and then stored over pellets of sodium hydroxide in a closed vessel in order to remove unsaturated compounds which might lead to resin formation. [Tobias and Gerard, 1941, claim that isodecane (2,7-dimethyloctane) is the index fluid of choice for use in small capillaries.]

The size of the chambers may be varied by filling them with paraffin to any chosen level. The surface of the paraffin is then smoothed out with the flat end of a metal rod. Both chambers are adjusted to about the some volume. A disc of Lucite with one depression to hold the biological material and another to hold the droplet of alkali used to absorb carbon dioxide is placed in each chamber.

Both chambers are charged in exactly the same manner with the

exception that the biological material is placed only in one of them. The index droplet is placed on the capillary by introducing a little kerosene into one of the drilled holes, and removing the excess by absorbing it into the end of a hardwood toothpick. Then the droplet is forced to the proper position and the capillary is clamped in place.

The assembled apparatus is tested for leaks by warming one end with the hand. When the hand is removed, the index droplet will return to its original position if no leaks are present.

Calibration. The volumes of the chambers are determined, after greasing the metal to prevent amalgamation, by filling with mercury, squeezing out the excess by pressing a glass plate over the chamber, and weighing the mercury left. The volumes of the Lucite discs are also determined by weighing them and dividing by the specific gravity of the Lucite. The volumes of the capillary posts and the capillary tubes are calculated from measurements of the dimensions of the holes.

Corrections for the volume of kerosene adhering to the capillary walls were found to be 0.45 ± 0.10% for a 0.57 mm. diameter capillary, and 0.42 ± 0.07 for a 0.22 mm. capillary, in experiments by Cunningham and Kirk (1940). The corrections were determined by measuring the length of a kerosene droplet before and after it was made to move along the capillary for a known distance. The volume of liquid on the walls, calculated from this measurement, divided by the total volume of the capillary traversed by the droplet times 100 gives the percentage error.

Calculations. Cunningham and Kirk (1940) gave the derivations of the formulae which may be used to calculate changes in the amount of gas in the apparatus. The change in the gas volume (ΔV_{P_0}) at the initial pressure (P_0), which equals the barometric pressure at the time the apparatus is sealed, is given by the following expression for the case of a reaction liberating carbon dioxide, as in the interaction of acid and bicarbonate in the reaction chamber:

$$\Delta V_{P_0} = nAd\left(\frac{V_c}{V_c - Ad}\right)$$

where n is the ratio of the total volume of the two chambers to the volume of the compensation chamber, A the effective cross-sectional area of the capillary (actual area minus 0.4% to correct for the film of kerosene which coats the wall), d the displacement of the index

droplet and V_c the volume of gas in the compensation chamber and capillary up to the index droplet. When Ad is negligibly small with respect to V_c, the expression may be reduced to:

$$\Delta V_{P_0} = nAd$$

Conversion of (ΔV_{P_0}) to standard conditions is carried out in the usual manner.

The preceding equations do not take into account the volume of gas which may be dissolved in the liquid. For carbon dioxide the following relation obtains at temperature t:

$$CO_2 \text{ (dissolved)} = \left(\frac{\Delta P_0}{V_R + Ad}\right) P_f \cdot \alpha\, CO_{2_t} \cdot Vl_R$$

where V_R is the volume of gas in the reaction chamber plus the volume in the capillary up to the index droplet. P_f is the total gas pressure in the reaction chamber, $\alpha\ CO_{2t}$ is the solubility coefficient of carbon dioxide for the liquid at temperature t and one atmosphere pressure of carbon dioxide (vol. gas at S.T.P./vol. liquid), and Vl_R is the volume of liquid in the reaction chamber.

If the precision of the measurements merits corrections for dissolved oxygen and nitrogen in the liquid, additional formulae may be applied. Thus the volume of oxygen or nitrogen forced into solution by compression of gas in the compensation chamber during the experiment equals:

$$\Delta O_2 \text{ (dissolved)} = \alpha\ O_{2_t} \cdot Vl_c \cdot P_{O_2} \left(\frac{P_f}{P_0} - 1\right) \qquad (a$$

$$\Delta N_2 \text{ (dissolved)} = \alpha\ N_{2_t} \cdot Vl_c \cdot P_{N_2} \left(\frac{P_f}{P_0} - 1\right) \qquad (b)$$

where $\alpha\ O_{2_t}$ and $\alpha\ N_{2_t}$ are the solubility coefficients of the respective gases in the liquid at temperature t, Vl_c is the volume of liquid in the compensation chamber, P_{O_2} and P_{N_2} are the initial partial pressures of the respective gases (in calculating these values one should not forget to consider the aqueous tension), and P_f is the final pressure of the system. These equations are based on the fact that the partial pressures of the gases in the compensation chamber have increased to (P_f/P_0) times their original values. Since the partial pressures of the gases in the reaction chamber have decreased to

$(V_R - Ad)/V_R$ times their original values, the volume of oxygen or nitrogen released from solution in the reaction chamber during the experiment equals:

$$\Delta O_2 \text{ (dissolved)} = \alpha O_{2_t} \cdot Vl_R \cdot P_{O_2} \left(\frac{VR - Ad}{VR} - 1 \right) \qquad (c)$$

$$\Delta N_2 \text{ (dissolved)} = \alpha N_{2_t} \cdot Vl_R \cdot P_{N_2} \left(\frac{VR - Ad}{VR} - 1 \right) \qquad (d)$$

These last quantities (*c* and *d*) are negative, but they tend to give rise to error in the same direction as the preceding ones (*a* and *b*). All four Δ values are added, and the sum is subtracted from ΔV_{P_0}. Correction for dissolved carbon dioxide is made by adding the volume that has dissolved to the value for ΔV_{P_0}.

In the case of respiration experiments the total pressure of the system decreases. Hence:

$$\Delta V_{P_0} = nAd \left(\frac{V_c}{V_c + Ad} \right)$$

Since only oxygen is removed during the respiration, the partial pressures of oxygen and nitrogen do not remain equal in the two chambers. In the compensation chamber, the partial pressures of both gases are reduced to $(V_c/[V_c + Ad])$ times their original value. On the other hand, the partial pressure of nitrogen in the reaction chamber has been increased to $(V_R/[V_R - Ad])$ times its initial value, while the partial pressure of oxygen has decreased to:

$$\left(\frac{V_R}{V_R - Ad + nAd} \right) P_{O_2}$$

Substitution of these factors for the partial pressure changes in the preceding formulae for the volumes of oxygen and nitrogen dissolved will permit the corrections to be applied.

(*b*) *Cunningham-Kirk Open-Tube Respirometer*

The capillary tube respirometer of Kalmus (1928) consists of an enlargement at one end of a capillary tube to serve as a reaction vessel while the other end of the capillary is open to the air. The position of the meniscus of liquid in the capillary is used to indicate

gas volume changes. The original technique of Kalmus has been the object of considerable criticism, but Cunningham and Kirk (1942)

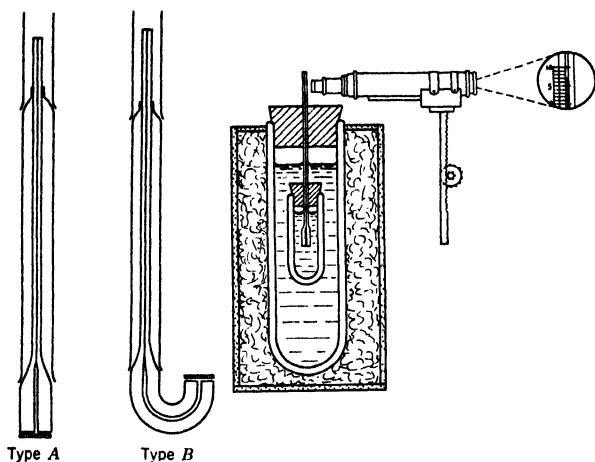



Fig. 96. Left: two capillary respirometers, shown with protective jackets of glass tubing. Right: respirometer assembly with microscope for observing shift of meniscus. *From Cunningham and Kirk (1942)*

have instituted improvements and they have chosen this open-tube instrument in preference to differential types for studies requiring a very high degree of sensitivity, *e.g.*, measurements of the respiration of a single *Paramecium*. The sensitivity obtained by Cunningham and Kirk was 5×10^{-5} μl. While the absolute accuracy is doubtful, the accuracy for relative values was given as at least $\pm 15\%$ for the instruments used. It should be pointed out that the technical difficulties involved in the use of this type of respirometer are very great, and these will, no doubt, seriously limit its application.

Diagrams of the instruments are given in Figure 96; their dimensions follow:

Dimension	Type A	Type B
Volume of liquid phase	3.0 μl.	13.0 μl.
Volume of gas phase	0.5 μl.	0.5 μl.
Diameter of respiration chamber	0.56 mm.	0.56 mm.
Diameter of manometer	0.08 mm.	0.08 mm.
Length of manometer	200 mm.	200 mm.

Double Dewar flasks are used to maintain constant temperature for the period of measurement (1–2 hr.). The outer flask is surrounded by a 1 in. layer of cotton packing; it has a 1 l. capacity and is used three fourths filled with water. The inner flask has a 4 oz. capacity and is two thirds filled with water.

The respirometer tube is cleaned by filling it with cleaning solution and after 30 min. it is rinsed with distilled water, followed by 0.01 *M* sodium bicarbonate, and finally distilled water again. It is then dried by drawing filtered air through it, and is allowed to stand overnight to eliminate any temperature differences within the glass.

A respiration experiment on a *Paramecium* is carried out as follows:

1. Fill the tube to within 4 mm. of the chamber end with 0.5 *N* sodium hydroxide by filling the cup at the top of the tube with alkali and allowing the tube to fill by gravity. When the solution reaches the proper position pour out that remaining in the cup.
2. Place a droplet of tap water containing the organism over the end of the respirometer chamber and force about 2 mm. of this tap water into the 0.56 mm. capillary by inserting a needle through the drop into the capillary and then withdrawing it.
3. Observe the end of the tube until the *Paramecium* enters it and then quickly wipe off the surface tap water and seal the end of the capillary with a piece of vaselined cover slip.
4. Fill the cup around the upper part of the tube with the sodium hydroxide soln. and place the respirometer in the Dewar flasks.
5. After 1 hr. remove the alkali from the cup with a fine pipette and plug the top of the cup with cotton.
6. After 1 hr. more focus the microscope (magnification 100×) on the meniscus of the liquid in the capillary and record the rate of fall of the meniscus. During these measurements, note the temperature and barometric pressure. If these vary sufficiently to result in significant errors, discard the experiment.

The errors introduced by changes in (*1*) temperature, (*2*) barometric pressure, (*3*) height of the liquid in the tube, (*4*) volume on dilution of the alkali soln., (*5*) volume of the dissolved gas phase with change in partial pressure, (*6*) bore of the capillary, and (*7*) surface tension of the liquid in the capillary, have been discussed by Cunningham and Kirk (1942). The original paper of these authors should

be consulted for the determination of each of these errors. For the two instruments described by them, the algebraic sum of those errors which are constant and independent of the presence of the organism in the respirometer equalled -1.6×10^{-4} µl./hr. for the type A, and

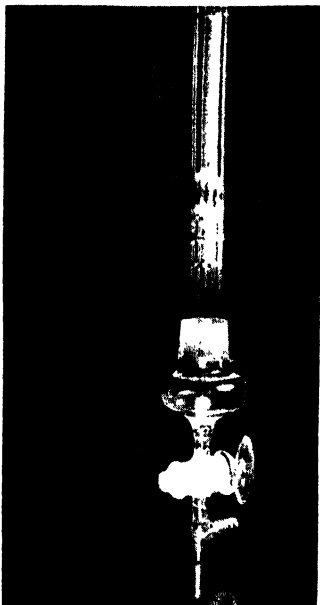



Fig. 97. Glass stopper with respirometer tubes attached. *From Tobias (1943)*

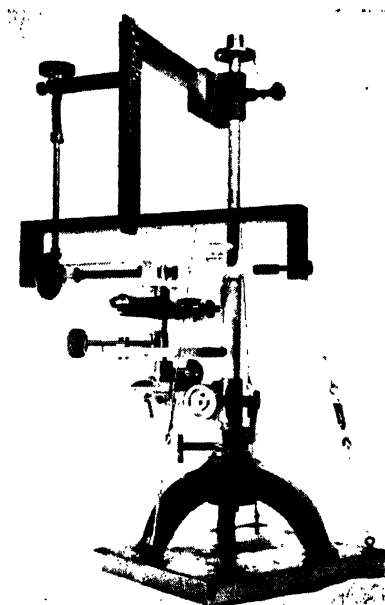



Fig. 98. Complete Tobias-Gerard respirometer assembly. *From Tobias (1943)*

-4.2×10^{-4} µl./hr. for the type B instrument. The summation of the errors which are dependent on the presence of the organism amounted to 0.147 v for the type A, and 0.407 v for the type B instrument, where v is the observed value for the volume of oxygen measured.

(*c*) *Tobias-Gerard Respirometer*

Tobias and Gerard (1941) employed the principle, used earlier by Gerard and Hartline (1934), of placing a small capillary reac-

tion chamber in a large compensation vessel which is sealed off to make the system independent of barometric changes. Volume changes within the capillary chamber are so small in comparison to the volume of the compensation vessel that these changes can be considered completely undamped, and the arrangement makes for great stability in measurement. An improvement initiated by Tobias and Gerard in this type of respirometer is the use of ten or more of the capillaries at the same time in a single compensation vessel. Gas volume changes of the order of 0.0005 to 0.001 microliter per minute can be followed at minute intervals with individual readings varying from the mean by about 8%. For five to ten minute intervals the accuracy is about 2%.

Apparatus. The respirometer is made by sealing a 90 mm. length of 0.2 mm. glass capillary tubing into a 30–40 mm. length of 1.2–1.5 mm. thin-walled glass tubing with DeKhotinsky cement. The wider tube serves as the reaction chamber while the capillary contains the index droplet by means of which gas vol. changes are measured. It has been found that isodecane (2,7-dimethyloctane) is superior to other index fluids for small capillaries. Bits of filter paper soaked with acid or alkali, and separated by dry paper guards, may be used in the reaction chamber to absorb certain gases during the reaction. The absorbing materials and the tissue are placed in the reaction tube. The open end of the latter is then sealed with plasticine. The isodecane droplet is placed in the capillary and the respirometer is mounted on a central thick-walled capillary tube connected to the glass stopper of the compensation vessel (Figs. 97, 98). A number of the respirometers may be mounted around the central tube as shown. The glass stopper bearing the respirometers is then fitted into the compensation chamber, which is supported horizontally. The entire glass unit is submerged in a thermostat bath. By rotating the stopper, individual capillaries can be brought into the field of the horizontal micrometer microscope employed to determine the positions of the index droplets. At the end of an experiment the small capillary is broken off and the diameter of the lumen at the end is measured with an ocular micrometer.

Certain mechanical improvements have been introduced (Tobias, 1943): "Permanent, heavy-walled capillary units, expanded at one end to accommodate tissue and absorbing reagents, replace the fine capillaries. A small ground-glass cap instead of plasticine closes the

chamber. The units are mounted in a vertical bank inside a brass-glass box. Brass clips into which the respirometers fit are rotatable from the outside of the box by means of a metal arm connected thereto by a packed joint. Since the tissue being studied clings to the roof of the expanded end of the unit, a droplet of substrate to be added may be placed on the floor along with a small lead shot coated with celloidin and paraffin. Rotation of a single capillary at any time then causes the mixing ball to drag the reagent around to the tissue. Greasing of the cap-respirometer joint makes it transparent and thus allows visual observation of the tissue being studied."

(*d*) *Scholander Micrometer Burette Differential Respirometers*

Scholander (1942a) adapted his micrometer burette (page 262) to the measurement of small gas changes in volumetric respirometers of his design. One of the respirometers was sensitive to about 0.3 μl. per hour and another to about 0.01 μl. per hour. Greater refinement would be achieved by reducing the dimensions of the micrometer spindle and the glass parts. In a subsequent publication Scholander and Edwards (1942) described a larger respiration chamber for an apparatus designed for measurements on aquatic organisms such as sand crabs, dragonfly larvae, or water plants. This instrument was sensitive to within 1 μl. per hour.

The principle of the apparatus lies in the maintenance of constant gas volume in a reaction vessel by the addition of oxygen from a micrometer burette to replace that used up by the respiration. The carbon dioxide evolved is absorbed by alkali. The pressure in the reaction vessel is balanced against that in a compensation vessel.

Respirometer Sensitive to 0.3 Microliter per Hour. This apparatus (Fig. 99) consists of a micrometer burette (*1*), storage bulb for oxygen (*2*), respiratory chamber (*3*), manometer (*4*), and a compensation vessel (*5*). The capillary bore is 1 mm. The frame holding the apparatus is held by a rod which passes through a hole (*7*), and it rests on the water bath at *6*. The biological material in *3* is separated from the carbon dioxide absorbent, which is in the bottom of the same vessel. A ground joint, or a syringe needle pushed through a rubber stopper as shown in *3*, may be used to connect the respiratory chamber with the rest of the apparatus.

Brodie fluid is placed in the manometer and water is used in the compensation vessel. A layer of water is also used to cover the mercury in *2*.

The chamber (*3*) is attached, while both stopcocks are open to the air. At the start of an experiment the stopcocks are set as shown in

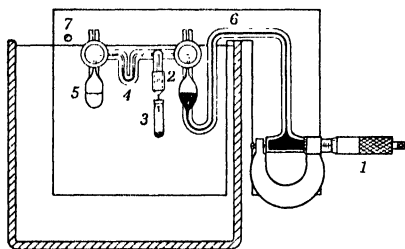



Fig. 99. Volumetric respirometer, sensitive to about 0.3 μl. per hour. *From Scholander (1942a)*

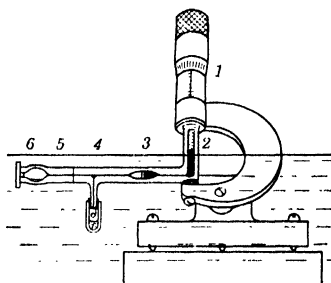



Fig. 100. Volumetric respirometer, sensitive to about 0.01 μl. per hour. *From Scholander (1942a)*

the figure; during the experiment the micrometer is used to keep the menisci level in the manometer tube by replacing the oxygen as it is consumed. In order to prevent contamination of the oxygen in *2*, the stopcock attached to the oxygen storage vessel is kept closed except when the micrometer adjustment is made.

Respirometer Sensitive to 0.01 Microliter per Hour. This apparatus (Fig. 100) consists of a micrometer whose spindle has been replaced by a $^1/_{16}$ in. diameter drill rod which passes through a fiber washer with an air-tight fit into the glass tube (*2*). The glass part is held in the micrometer frame. Oxygen is stored at *3*; the respiratory chamber fits on the side arm at *4*; the indicator drop is at *5*, and the compensating chamber at *6*. The capillary bore is about 0.25 mm. The micrometer is screwed to a heavy brass foot as illustrated.

The apparatus is first filled with mercury and carefully freed from air bubbles. The reservoir (*3*) is filled with oxygen after a little water is drawn in to cover the mercury. The respiration chamber is then connected; it contains a loop of platinum wire to hold the sample in a drop of medium while the carbon dioxide absorbent is

placed on the bottom of the vessel, or the absorbent may be placed in the loop and the sample on the bottom.

An index drop of 2% Turgitol, or other wetting agent, is introduced through the compensation chamber with a hypodermic needle. Then a drop of water is placed in the compensation chamber, and finally the opening is greased and sealed with a flat piece of glass. The instrument is operated in a manner similar to that employed with the larger apparatus.

2. Gas Analysis

(a) *Scholander Micrometer Burette Gas Analyzer*

Scholander (1942b) employed his micrometer burette to develop an apparatus by means of which 10 μl. of a gas mixture may be analyzed for its various components with an accuracy of about 0.1% of the total sample. Specific absorbents for each component are used, and the volume change resulting from the absorption of each gas is measured.

Apparatus. The apparatus is shown in Figure 101 (available from O. Hebel, Edward Martin Biological Laboratory, Swarthmore College). The micrometer spindle is replaced by a $^1/_{16}$ in. drill rod (*2*), which displaces about 50 μl. by its full traverse. Each micrometer scale division corresponds to approximately 0.02 μl. and estimates are safely made to 0.005 μl. The capillary bore is around 0.25 mm. and the bulb (*3*) has a capacity of about 50 μl. The absorption chamber (*1*) has a bore no greater than 2.5 mm. The fine line (*4*) is used for reading. The drill rod must fit perfectly tight through a fiber washer (*5*) and the mercury vessel must be completely freed from air. The waterjacket surrounding the burette is clamped so that it can be tilted by the handle (*6*).

Scholander uses separate 2 ml. syringes to hold the respective liquids required, *i.e.*, mercury, a manometer liquid of 2% Turgitol (wetting agent), 0.5 *N* sulfuric acid, and gas absorbents such as 0.25 *N* potassium hydroxide which has been shaken well with air (for carbon dioxide) and hydrosulfite (for oxygen). (The latter is prepared by adding a mixture of 10 parts hydrosulfite and 1 part sodium anthraquinone-β-sulfonate to a small test tube filled with 0.25 *N* potassium hydroxide, containing a drop of mercury for mix-

ing, until a dark red solution is obtained. The full tube is closed, taking care to exclude air bubbles, shaken, and finally stored in a syringe with a bubble of nitrogen.) Each syringe is connected by stiff rubber tubing to a drawn-out glass tube. The orifice of the glass

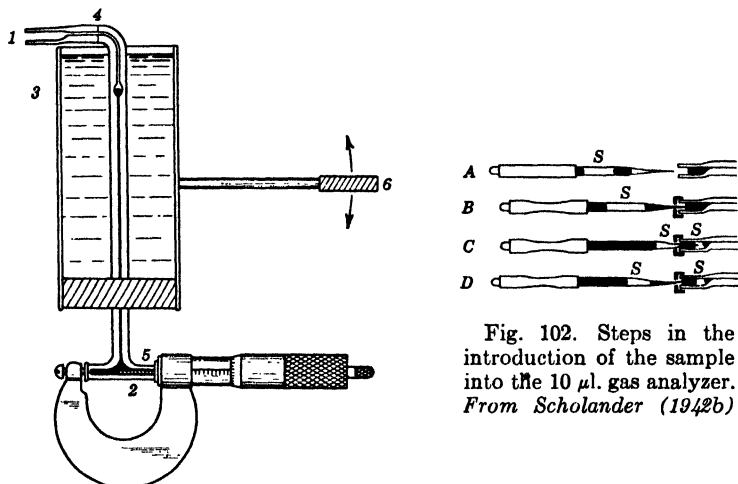



Fig. 101. Analyzer for 10 μl. of gas mixture. *From Scholander (1942b)*

Fig. 102. Steps in the introduction of the sample into the 10 μl. gas analyzer. *From Scholander (1942b)*

tip should not be fine enough to cause pressure to develop in the syringe during delivery, since this would force air or nitrogen into solution and result in low absorption values. A glass tip connected to suction is also required. Syringe needles, if not finer than 20 gage, may be substituted for the glass tips.

The transfer pipette (Fig. 102) is convenient for handling the gas sample (S). It consists of 2–2.5 mm. bore glass tube drawn out at one end and connected to a piece of rubber tubing at the other. One end of the rubber tube is plugged with a piece of glass rod. Mercury is used to confine the sample as in A. A transparent cap with a central hole fits over the absorption chamber to guide the tip of the pipette.

Manipulation. As an example of the procedure to be followed, consider the analysis of a mixture of carbon dioxide, oxygen, and nitrogen:

1. Rinse the absorption chamber and bulb with 0.5 N sulfuric acid and move the mercury up to the absorption chamber.

2. Introduce the gas sample (about 10 μl.) into the absorption chamber following the steps shown in Figure 102. Guide the tip of the pipette so that it does not touch the wall of the chamber during the transfer.

3. Draw the mercury back into the capillary until the meniscus is at the mark, and then take the first micrometer reading.

4. Draw the gas into the capillary, and after it a little mercury.

5. Suck away the mercury left in the absorption chamber and replace it with manometer fluid.

6. Expel the residual mercury in the capillary into the manometer fluid contained in the absorption chamber. Draw in a little of the fluid, and suck away that remaining in the chamber. Leave only 2 mm. of the fluid in the capillary.

7. Bring the gas-fluid meniscus to the mark and take a second reading. The volume of the sample is obtained from the difference between the two readings.

8. Move the manometer fluid out into the absorption chamber again and fill the chamber with the potassium hydroxide solution to join the fluid without enclosing any air bubbles.

9. Move the gas sample out into the alkali in the chamber until the gas bubble breaks loose. Then move the bubble back and forth by tipping the instrument.

10. Draw the bubble back into the capillary and take a third reading when the gas-mercury meniscus reaches the mark.

11. When the last of the gas has been drawn into the entrance of the capillary, replace the alkali by manometer fluid and then suck all of the fluid away except a short drop in the capillary.

12. Move the fluid-gas meniscus to the mark and take a fourth reading. The volume of the sample minus the carbon dioxide is obtained from the difference between the third and fourth readings.

13. Repeat steps 8–12 using the hydrosulfite solution to absorb the oxygen. Allow 2 min. for the oxygen to be absorbed. The gas remaining after this absorption is entirely nitrogen.

(*b*) *Berg Simplified Gas Analyzer*

Using the principle of the Scholander micrometer-burette gas analyzer, Berg (1946) employed the simplified apparatus illustrated in Figure 103. The thermometer tubing is 6 in. long and has a bore of 0.11 mm. The water jacket surrounding it affords protection

against temperature change during the analysis. A saturated solution of lithium chloride, preferably equilibrated with gas of approximately the same composition as the sample to be analyzed, is used to fill the apparatus. The gas sample is introduced into the absorption chamber while the instrument is held vertically with the chamber end down. By opening the screw clamp on the rubber tubing attached to the capillary, the gas is drawn into the latter and the volume is determined by noting the length of the gas column and multiplying it by the cross-sectional area (0.0095 mm.^{2}). The liquid in the absorption chamber is then replaced by the gas-absorbing solution (lithium chloride containing potassium hydroxide for carbon dioxide, and alkaline pyrogallol for oxygen) and the gas is forced out into the absorption chamber. The unabsorbed portion of the gas is then drawn back into the capillary and its volume is

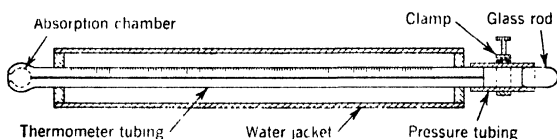



Fig. 103. Simplified gas analyzer. *From Berg (1946)*

measured. To be sure of complete absorption the gas is re-expelled into the absorption chamber and after a number of seconds redrawn into the capillary. This process is repeated until no change in the volume of the residual gas is observed.

The small bore of the capillary enables the analysis of samples in the range 0.4–1 μl. Under the most favorable conditions the experimental error can be reduced to $<0.3\%$. The chief source of error is the diffusion of the gas into the lithium chloride solution and hence the need for saturating this solution with gas of approximately the same composition as that of the sample to be analyzed. There might be an advantage in using mercury as the analyzer fluid in a manner similar to that employed by Scholander with the micrometer-burette apparatus.

(c) *Scholander-Roughton Syringe Gas Analyzer*

The syringe gas analyzer, first described by Scholander and Roughton (1942), has been used primarily for the estimation of gases in blood samples of the order of one drop. Analysis can also be performed on other fluids, and, in general, the apparatus may be used for the analysis of components in gas mixtures. As applied to

blood gases, oxygen and carbon monoxide can be determined in 40 μl. blood samples with an accuracy of 0.15 to 0.20 volume per cent, nitrogen in 120 μl. samples to 0.05 volume per cent, and carbon dioxide in 13 μl. samples to about 1 volume per cent. The time required for a determination is 6–10 min. In these methods the blood is drawn into a capillary attached to a 1 ml. tuberculin syringe, the gas is liberated by a suitable reagent, the gas volume is noted, specific absorbents are used to remove the gases separately, and the volume change produced by the removal of each component is recorded.

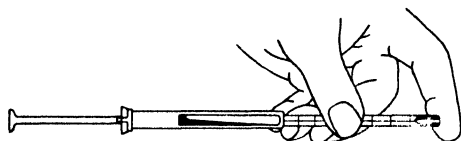



Fig. 104. Shaking of syringe and extraction of gas.
From Roughton and Scholander (1943)

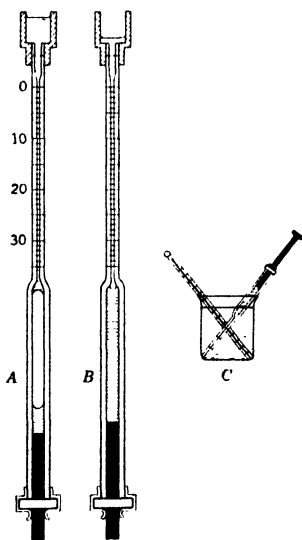



Fig. 105. *A* and *B*, syringe showing the technique for absorption of the carbon dioxide used for extraction. *C*, temperature equilibration of gas bubble in capillary before the reading is made. *From Roughton and Scholander (1943)*

The apparatus, which was slightly modified by Roughton and Scholander (1943), consists of a 1 ml. Pyrex tuberculin syringe with an arresting clip on the plunger to prevent its slipping, and with a standard bore precision 0.5 mm. Pyrex capillary fused to its nozzle (Fig. 105). The top of the capillary is expanded to a cylindrical cup of about 1.5 cm. length and 2.5 mm. bore. The capillary, which is

7–8 cm. long, is graduated into thirty divisions, each 2 mm. long. A detachable rubber cup of about 1 ml. capacity may be fitted over the cylindrical glass cup.

The sampling pipette consists of a piece of thin-walled glass tubing having a 1–1.5 mm. bore, and it is drawn out to a tip which is ground smooth in order to permit a snug fit into the bottom of the glass cup. The mark on the pipette is placed so that the volume from the mark to the tip is equal to 100 divisions on the capillary, *i.e.*, 39.3 μl. The capillary and the pipette can be calibrated with a microburette or by mercury weighing. (Interchangeable pipettes and syringes are supplied by Mr. J. D. Graham, Department of Physiology, University of Pennsylvania Medical School.)

Collecting Blood Samples. Citrated blood samples may be collected directly in the blood pipettes from finger pricks as described by Scholander (1942c). A short tube, 15 mm. long, flanged out at one end is held on the finger tip by a rubber band which presses the flange against the skin. A few crystals of citrate are placed in the tube; a tourniquet is wound around the finger toward the tip. The finger is pricked through the tube using a spring blood lance, and blood is quickly drawn into the pipette from the bottom of the tube.

The blood may be collected anaerobically in syringes and transferred to the pipettes. The transfer is made by setting the pipette on a table with the tip protruding about 1 in. over the edge, pressing the tip into the opening of the syringe nozzle while the syringe is held horizontally, and then slowly filling the pipette by screwing in the plunger (Roughton and Scholander, 1943).

OXYGEN

Roughton and Scholander (1943) described the oxygen method in which an excess of carbon dioxide is used to extract the oxygen, the carbon dioxide is then absorbed in alkali, and the volume of the residual gas is measured in the capillary before and after the oxygen is absorbed by alkaline pyrogallol.

Roughton and Scholander Method for Oxygen

SPECIAL REAGENTS

Caprylic Alcohol.

Ferricyanide Solution. Dissolve 12.5 g. potassium ferricyanide, 3 g.

potassium bicarbonate, and 0.5 g. saponin in distilled water and make up to 50 ml. Do not use the soln. after 3 days.

Acetate Buffer. Dissolve 70 g. sodium acetate ($NaC_2H_3O_2.3H_2O$) in 100 g. distilled water and add 15 ml. glacial acetic acid.

45% Urea (used as cleaning solution).

10% Sodium Hydroxide.

Pyrogallol Solution. Add 15 g. powdered pyrogallol to 100 ml. 20% sodium hydroxide under a 2 cm. layer of oil. Dissolve the pyrogallol under the oil by stirring with a glass rod.

PROCEDURE

1. With the syringe analyzer in a vertical position and the plunger pushed up, fill the cup with ferricyanide soln. Draw the soln. down to the bottom of the syringe, push it back up into the cup, and suck it out. Repeat twice with fresh lots of ferricyanide, taking care to avoid trapping air bubbles. Leave the dead space in the syringe full of ferricyanide. Use no grease or oil in the syringe.

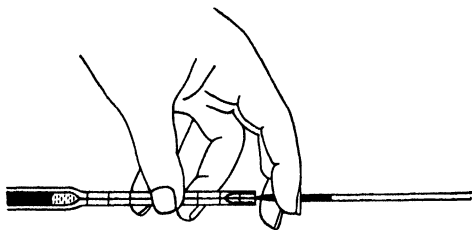



Fig. 106. Transfer of blood from pipette directly to capillary. *From Roughton and Scholander (1943)*

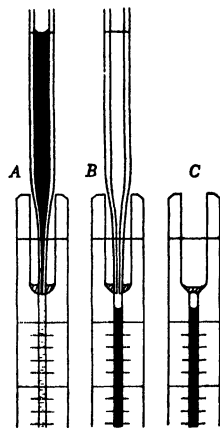



Fig. 107. Further details of the transfer of the blood from the pipette to the capillary. *From Roughton and Scholander (1943)*

2. Fill the glass cup to the mark with ferricyanide soln. and draw it down to the bottom of the cup.

3. Place a drop of caprylic alcohol on the bottom of the cup.

4. Draw the sample from the pipette, which has been filled to the mark (about 40 μl.) into the capillary as shown in Fig. 106. Pull out the plunger gradually so that the sample is slowly and evenly drawn in followed by an air bubble of about 1 mm. length (*A* and *B*, Fig. 107). The air bubble prevents the blood from getting back up into the pipette. If the pipette tip is properly ground and the correct pressure applied during the transfer, no appreciable quantity of caprylic alcohol will be drawn into the capillary.

5. Remove the pipette quickly, and expel the air bubble (*C*, Fig. 107) through the caprylic alcohol, using a fine wire if necessary or tapping the capillary.

6. Draw in a column of the caprylic alcohol two divisions in length onto the top of the blood and suck out the rest of the caprylic alcohol from the cup.

7. Fill the cup to the mark with acetate buffer and draw it down to the bottom of the cup.

8. Immediately fill the cup to the top with 45% urea and close firmly with the finger.

9. Shake the closed apparatus vigorously for 2 min. in a horizontal position, gradually pulling out the plunger as the gases are evolved to keep the gas pressure in the syringe about 1 atmosphere. About 0.75 ml. is usually evolved (Fig. 104). The amount of carbon dioxide evolved may be varied by changing the strength of the bicarbonate in the ferricyanide soln.

10. Carefully release the finger while adjusting the plunger to keep the gas meniscus in the capillary. Let a little urea soln. run down into the capillary, and allow it to remain there until the walls are perfectly clean.

11. Remove three fourths of the urea soln. from the cup. Fit the rubber cap over the glass cup, and fill it with 10% sodium hydroxide without trapping air bubbles (*A*, Fig. 105).

12. Draw a little of the alkali into the syringe. As the carbon dioxide is absorbed more alkali will be sucked in until only a small bubble of the other gases will remain (*B*, Fig. 105). The absorption requires a few sec., and, before it is complete, carefully move the residual bubble into the capillary to prevent reabsorption of oxygen.

13. Remove the rubber cup. Empty the glass cup, and set the capillary in a beaker of water at room temperature for 30 sec. (*C*, Fig. 105).

14. Remove from the water, dry by lightly wiping, taking care not to handle the capillary, and read the gas vol. (V_1) in divisions.

15. Fill the glass cup with pyrogallol soln. and absorb the oxygen by pulling the gas down to the bottom of the capillary and back again a few times. Finally move the gas bubble very slowly up to the top part of the capillary. Equilibrate the temperature, and again read the vol. (V_2) in divisions. When V_2 is only a few divisions, the second temperature equilibration may be omitted.

16. Run a blank on the reagents by omitting the sample.

17. Calculate the oxygen content from the formula:

$$\text{Oxygen} = (V_1 - V_2 - C)f$$

where C is the blank correction for oxygen in the reagents, and f is the correction factor for temperature, aqueous vapor tension, and barometric pressure. C amounts to 1.0 to 1.1 volume per cent at room temperature as a rule; f may be obtained from the usual tables such as that given by Peters and Van Slyke (1932, page 129, Table 15).

CARBON MONOXIDE

Scholander and Roughton (1943a) described three applications of the syringe analyzer to the measurement of carbon monoxide in blood: a general method for saturation ranging from 0–100% carbon monoxide hemoglobin, a method for the measurement of both oxygen and carbon monoxide on the same sample of blood, and a method sufficiently precise for the determination of blood volume in which the carbon monoxide level is held below 2 volume per cent. Only the first of these three will be described.

Scholander and Roughton Method for Carbon Monoxide

SPECIAL REAGENTS

Winkler Solution. Place 20 g. cuprous chloride, 25 g. ammonium chloride, and 75 g. water in a bottle just large enough to contain them. Stopper the bottle, shake with as little air as possible, and allow the precipitate to settle. Put a coil of copper wire in the soln. and cover the liquid with a layer of paraffin oil. The reagent becomes almost colorless after a while.

Other Reagents. The other reagents required are those listed for the oxygen method (page 331) with the exception of the 10% sodium hydroxide. The distilled water is aerated.

PROCEDURE

1–10. Same as for oxygen (pages 332-333).

11. Substitute pyrogallol soln. for the 10% sodium hydroxide, but otherwise same as for oxygen (page 333).

12–14. Same as for oxygen (pp. 333–4). Read vol. of bubble (V_1).

15. Flush the glass cup clean with water and leave filled. Quickly pull about three fourths of the water in the cup down into the syringe to form a layer over the blood mixture. Then immediately run the bubble with the clean water below it up into the top of the capillary.

16. Empty the water from the glass cup and fill it with Winkler soln.

17. Incline the syringe so that the cup points downward at a slight angle from the horizontal. Cautiously screw in the plunger to drive the gas bubble out into the glass cup where it can rest near the junction of the capillary and the cup. Suck in the Winkler soln. behind the bubble to half fill the capillary, and rotate the instrument gently for a few sec. to complete the absorption of the carbon monoxide. Then turn the syringe to the vertical position with the cup down and suck the gas bubble back into the capillary. Measure the gas vol. (V_2) in the usual fashion, and calculate the carbon monoxide content from the formula:

$$\text{Carbon monoxide} = (V_1 - V_2)f$$

where f is the correction factor (page 334).

NITROGEN

The adaptation of the syringe analyzer to the measurement of nitrogen in fluids was made by Edwards, Scholander, and Roughton (1943). Their method calls for a pipette with two marks, calibrated to deliver not only the usual volume (page 331) but also three times this. The principle of the method depends on extraction of all the nitrogen and part of the oxygen from the sample with carbon dioxide, absorption of the oxygen and carbon dioxide with alkaline hydrosulfite, and measurement of the nitrogen which remains.

Edwards, Scholander, and Roughton Method for Nitrogen

SPECIAL REAGENTS

Bicarbonate Solution. Dissolve 11 g. potassium bicarbonate in 100 g. water.

Acid Phosphate Buffer (about 5 *M*). Dissolve 95 g. sodium dihydrogen phosphate ($NaH_2PO_4.H_2O$) in 100 g. warm water.

Hydrosulfite Solution. To 50 ml. of 20% potassium hydroxide add 15 g. of a mixture of 10 parts sodium hydrosulfite and 1 part sodium anthraquinone-β-sulfonate. Store well stoppered in contact with as little air as possible.

45% Urea.

Caprylic Alcohol.

Aerated Distilled Water.

PROCEDURE

1. Same as for oxygen (page 332) substituting the bicarbonate soln. for the ferricyanide.

2. Dry the glass cups with cotton or filter paper and place a drop of caprylic alcohol in the bottom of the cup without trapping air bubbles.

3–5. Same as steps 4–6 in oxygen method (page 333). Take care to prevent caprylic alcohol from being drawn down into the capillary with the sample. A 120 μl. sample is used.

6–9. Same as steps 7–10 in oxygen method (see page 333) substituting the acid phosphate buffer for the acetate. Draw the urea soln. down to the bottom of the capillary but do not let it enter the syringe barrel.

10. Holding the syringe vertically, attach the rubber cap, and add about 1 ml. hydrosulfite soln. without trapping air bubbles in the glass cup.

11. Draw a little hydrosulfite into the syringe; the vacuum created by the gas absorption will draw in the rest of the soln. required for the complete absorption of the carbon dioxide and oxygen.

12. Push the bubble up into the lower part of the capillary; suck out the hydrosulfite from the rubber cap and detach the latter. Fill the glass cup with water, and draw three fourths of it down over the bubble.

13. Push the bubble up into the clean capillary very gently. Equilibrate the temperature and read the volume (V_1) in divisions (steps 13, 14, pages 333 and 334).

14. Run a blank on the reagents by substituting aerated distilled water for the blood; however, use one third the vol. of water, *i.e.*, to the first mark on the pipette (about 40 μl.). The blank then equals $V - (a/f)$ where V is the uncorrected nitrogen reading, a is the solubility of atmospheric nitrogen in water at room temperature in volume per cent (at 22°, $a = 1.2$), and f is the correction factor (page 334). The authors of the method found the blank to be constant and of the order of 1.3 to 1.5 units on the capillary, depending on the instrument used.

15. Calculate the nitrogen content of the blood from the formula:

$$\text{Nitrogen} = (V_1 - C)\ (f/3)$$

where C is the blank correction for the nitrogen in the reagents.

CARBON DIOXIDE*

Certain changes in the syringe analyzer technique were introduced by Scholander and Roughton (1943b) to enable the estimation of carbon dioxide. The gases are vacuum extracted from the sample mixed with acid buffer, and the gas volume is measured before and after absorption with alkali.

This determination requires a rubber-tipped wooden plug, made by dipping the end of a round toothpick in rubber latex, leaving a drop on the tip, and drying it, tip downward, at a moderate temperature in an oven (*A*, Fig. 108). A spacer for holding out the syringe plunger in a fixed position (*B*, Fig. 108) is also required. It consists of a piece of light sheet metal, about 1.5 cm. wide and 5.5 cm. long, folded into a V-shaped channel. The length of the spacer allows a gas space of about 0.75 ml. in the syringe.

Scholander and Roughton Method for Carbon Dioxide

SPECIAL REAGENTS

Carbon Dioxide-Free Distilled Water. Boil the water after adding a drop of sulfuric acid.

Caprylic Alcohol.

* See Bibliography Appendix, Ref. 52.

Acid Phosphate Buffer. Same as for nitrogen (page 336). Carbon dioxide has a low solubility in this soln.
10% Sodium Hydroxide.
Glycerol.

PROCEDURE

1. Lubricate the rear part of the dry plunger with a few streaks of glycerol and place it in the syringe barrel which is moist with water.
2. Fill the glass cup with distilled water, draw it one fourth down the barrel, and expel it through the cup. Leave the dead space in the syringe full of the water without trapping any air bubbles.
3. Pull out the plunger slightly so that the water meniscus at the bottom of the cup is lowered 1–2 mm. down into the capillary.
4. Hold the pipette against the opening of the capillary, trapping a small air bubble, and draw the sample (13 μl.) down very slowly into the capillary with the air separating it from the water.

5a. With the meniscus of the sample at the 30 or 35 mark, detach the pipette and suck out the sample from the cup. Slowly move the upper meniscus of the sample to the zero mark, and read the amount of sample *b* (page 340) in divisions.

5b. As an alternative procedure, move the sample down to a mark made at 33.3 divisions. Adjust the upper meniscus exactly to the zero mark with a fine suction tip, keeping the lower meniscus at the 33.3 mark. (In this way one third the normal pipette load is used and the carbon dioxide vol. has only to be multiplied by 3 to give the carbon dioxide content in volumes per cent after corrections for temperature, etc.)

6. Deposit a drop of caprylic alcohol on the bottom of the cup and eject the bubble of air above the sample through the caprylic alcohol with the aid of a piece of fine wire if necessary.
7. Same as step 6 in the oxygen method (page 333).
8. Fill the glass cup to the mark with acid phosphate and draw it down very slowly until the upper meniscus is 2 mm. below the bottom of the cup.
9. Moisten the rubber end of the wooden plug with phosphate buffer and, with a few drops adhering to it, insert it in the bottom of the cup, trapping a small air bubble.
10. With the plug resting loosely against the bottom of the cup, gently move the air bubble up until it touches the rubber tip. Then

press the plug against the bottom of the cup, leaving the bubble in contact with the rubber (*A*, Fig. 108). Keep the capillary closed in this way with the left hand throughout steps 11–17.

11. Place fresh glycerol in the plunger bearing.

12. Place one end of the metal spacer around the plunger under the plunger head, and hold it there with the other end sticking out at an angle. Keep the cup end of the syringe pointing upwards at a slant through the steps 13–16.

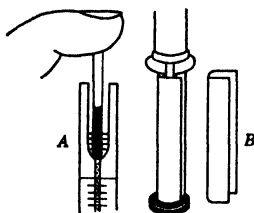



Fig. 108. *A*, Rubberized wooden plug for vacuum-sealing the glass cup. *B*, spacer for keeping the plunger extended in a fixed position during the vacuum extraction. *From Scholander and Roughton (1943b)*

13. Slowly move the plunger out so that the fluid meniscus under the stopper moves down the capillary very slowly. When the capillary and its opening into the barrel are drained of fluid, slowly draw the plunger out and move the free end of the spacer in until it fits against the syringe barrel (*B*, Fig. 108).

14. Add glycerol to the plunger bearing.

15. Shake the syringe for 2 min. with the cup end up to prevent fluid from blocking the opening to the capillary. Should the capillary become occluded, clear it by warming the capillary with the hand. Should foaming occur, release the plunger and draw it out again.

16a. With the capillary free from fluid, pull out the plunger to allow the spacer to fall out. Allow the plunger to rise rather rapidly until the lower meniscus is inside the capillary and the gas pressure is atmospheric.

16b. Should fluid bridge the opening to the capillary while the plunger is let in, adjust the speed of the plunger so that the bridge moves slowly up the capillary to enable proper drainage to occur.

17. Remove the plug and move the upper meniscus slowly and evenly to the zero mark when the gas bubble is at atmospheric pressure.

18. Equilibrate the temperature and read the vol. (V_1) in divisions as for oxygen (steps 13–14, pages 333–4). If liquid is in the capillary, subtract the length of the liquid column from the total reading.

19. Fill the cup with water and draw three fourths of it into the syringe. Return the bubble to the capillary with water beneath it.

20. Fill the cup with 10% sodium hydroxide, with the cup pointing downward and the bubble expelled into the alkali, some of which is drawn into the capillary as soon as the bubble is free.

21. To complete the carbon dioxide absorption, rotate the instrument a few times with the cup pointing slightly downward. Then with the instrument held vertically, cup down, suck the gas bubble back into the capillary.

22. Again adjust the temperature and take a gas vol. reading (V_2).

23. Calculate the carbon dioxide content in volume per cent from the equation:

$$\text{Carbon dioxide} = (V_1 - V_2)f(100/b)i$$

where b is the vol. of sample (step 5a), f is the gas correction factor (page 334), and i is an empirical factor correcting for reabsorption and incomplete extraction. For measurements on blood, $i = 1.015$.

3. Membrane Interferometer Volumetry

A sensitive indicator of pressure change which is based on the principle of the interferometer was developed by Tobias (1942). While the instrument has not yet been employed in biological experimentation, a brief description will be given nevertheless, since its further development and exploitation may be stimulated by so doing. The instrument responds to pressure changes of the order of 0.05 mm. water, or about 0.004 mm. mercury. It consists of a polished microscope slide on which an ordinary cover slip (0.1 mm. thick), having a hole ground through it about 2 mm. in diameter, is fastened with heavy shellac. A film of collodion is placed over the cover slip and then another cover slip with a hole drilled through it is fitted on top so that the holes of both cover slips coincide. Shellac is also used to fix the positions of the film and the top cover slip. The assembly is shown in Figure 109. When placed on the stage of a low-power microscope, and illuminated with monochromatic light,

displacement of the film with respect to the glass slide can be followed by recording the magnitude of the shift of the interference fringes produced. A movable ocular micrometer may be used to measure this shift.

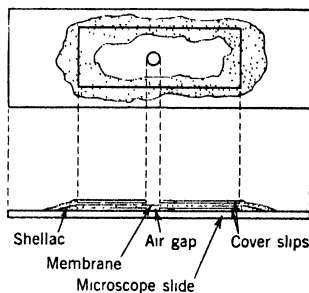



Fig. 109. Membrane interferometer manometer.
From Tobias (1942)

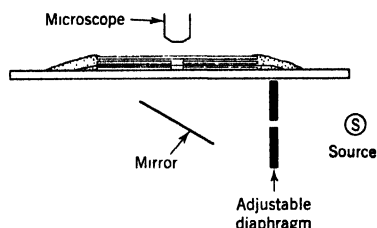



Fig. 110. Detail of membrane interferometer manometer, showing that fringes may be obtained by reflected light by shifting source, diaphragm, and mirror from below to above the interferometer.
From Tobias (1942)

The sensitivity of the instrument may be increased by employing shorter wavelengths of light. Tobias used the green mercury line at 546 mμ after the light was passed through a Corning filter. The use of a diaphragm to narrow the light beam lends sharpness to the fringes. By placing a glass slide over the top cover slip, a differential manometer with closed chambers on each side of the membrane is formed (as in Fig. 110). One of the chambers may be used as a reaction vessel, or, as water may condense on the membrane, it might be preferable to make connection between the chamber under the film and a reaction chamber ground out of the slide a short distance away by cutting out a segment in the cover slip to form a capillary channel between the two.

Drilling Holes in Cover Slips. A pile of ten to twelve cover slips are mounted in pitch on a heavy glass plate. Round holes are drilled with a carborundum or silica pencil bit operated in a hand drill using water as the lubricant. The pitch is removed by soaking in xylol for a day. The serrated edges are no handicap, but they can be rounded off by coating with several layers of a plastic.

Preparation of Membranes. The collodion films are made from a mixture containing 25 ml. celloidin (*Merck*), 25 ml. amyl

acetate, and 0.5 ml. *n*-butyl phthalate. The thinnest film that Tobias used and measured was about 20 mμ thick, although thinner films proved adequate.

B. MANOMETRIC

Of the two manometric techniques which will be considered here, Cartesian diver and optical lever manometry, the former holds the more prominent position. The relative simplicity of the apparatus and the great sensitivity and high precision of which it is capable make the diver technique one of choice for many gasometric studies on the histo- and cytochemical level. The diver has already been applied to the gasometric determination of isolated enzymes and other metabolic catalysts as well as to respirometry. Future developments can be expected to exploit extensively the many possibilities of this versatile technique.

1. Cartesian Diver Manometry

The application of the Cartesian diver to the measurement of the volume changes in small volumes of gas was conceived and first elaborated by Linderstrøm-Lang (1937b). A short discussion of the technique appeared in a subsequent report by Linderstrøm-Lang and Glick (1938). Certain technical changes were employed by Boell, Needham, and Rogers (1939) and Boell, Koch, and Needham (1939) in their noteworthy investigations dealing with the respiration and anaerobic glycolysis of regions of the amphibian gastrula. Other technical modifications were suggested by Rocher (1942, 1943). Theoretical aspects of Cartesian diver micromanometry have been given a complete treatment by Linderstrøm-Lang (1942, 1943). Following a study by Linderstrøm-Lang and Holter (1942) of the diffusion of gases through liquid seals in the diver, Holter (1943) presented a finely delineated description of diver technique which contains a full complement of precise detail, and includes the refinements and innovations subsequently introduced. Zeuthen (1943) designed divers of even smaller capacities and worked out the details of their use.

The principle of the diver technique lies in the fact that any change in the amount of the gas in the diver, which is used as a

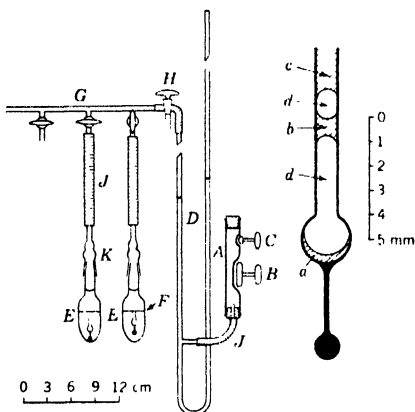
reaction vessel, requires a corresponding change in the pressure necessary to hold the gas volume constant so that the diver will remain submerged at a fixed level in the flotation medium surrounding it. Thus the pressure changes become measures of the changes in the amount of gas in the diver.

Divers having gas volumes of 1–10 μl. are referred to as "μl.-divers" by Holter (1943), and he calls the methods which employ them "μl.-methods." This nomenclature has obvious advantages over the use of ambiguous terms such as "ultramicro," "submicro," etc.

Fig. 111. Schematic drawing of measuring apparatus and diver.

Apparatus:
- *A*, rubber tubing
- *B*, coarse screw
- *C*, fine screw
- *D*, manometer
- *E*, flotation vessel
- *F*, circular mark
- *G*, connecting manifold
- *H*, three-way tap
- *J*, rubber pressure tubing
- *K*, ground-glass joint.

Diver:
- *a*, bottom drop
- *b*, neck seal
- *c*, mouth seal
- *d*, gas phase.

From Holter (1943)

(*a*) *Microliter Diver Technique**

A diagram of the diver and apparatus is shown in Figure 111 (diver equipment may be obtained from E. Petersen, Carlsberg Laboratory). The reaction mixture (*a*) is placed in the bottom of the diver, and gas evolution or absorption changes the amount of gas confined under the neck seal (*b*). Gas changes cause the diver to rise or sink in the flotation medium in the vessels (*E*), and the water manometer (*D*) is adjusted to vary the air pressure over the surface of the flotation medium in order to bring the divers to the mark (*F*) chosen as the equilibrium position. In this fashion the diver is used as a constant-volume gasometer.

* See Bibliography Appendix, Ref. 46.

Since the precision of the pressure measurement is about 1 mm. water, which corresponds to 0.0001 of the total pressure of 1 atmosphere, and the total volume of the type of diver commonly used is about 10 μl., the accuracy of the measurement of the gas volume in the diver is about 0.001 μl. Holter has pointed out that for profitable work with these divers, the volume changes to be measured should be of the order of 0.01 μl. per hour.

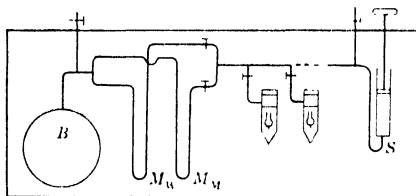
(1) TEMPERATURE CONTROL

It is advantageous to carry out work with divers in a room the temperature of which is not very different from that of the thermostat used with the diver apparatus. While not absolutely necessary, additional advantages are obtained if the room has an approximately constant temperature, since only the flotation vessels and the air bottle, which is connected to one end of the manometer, can be conveniently submerged in the thermostat. The manometer itself and the connecting tubing are subject to variations in the room temperature. Rocher (1942,1943) employed the arrangement shown in Figure 112. The manometers are submerged in the thermostat, and a cathetometer is used to take their readings.

The thermostat used for the apparatus in the Carlsberg Laboratory is maintained at a temperature 2° above that of the room, which is kept at 21°, and the thermostat is regulated with a precision of 0.01°. The stirrer for the thermostat water must run very quietly and it should not be attached to the thermostat itself in order to prevent its vibrations from being transmitted to the diver. A 40 watt lamp may be used as the heating element for a thermostat of the size used at the Carlsberg Laboratory (58 $\times$ 30 $\times$ 30 cm.). The light from the lamp should be shaded from the eyes of the operator. High-capacity heaters are to be avoided, since they continue to supply heat for a time after the current is cut off. Lamps are by far the best heaters.

Since the diver must be observed during the experiment, it is necessary that the front wall of the thermostat be made of glass, and the lighting must also be considered. A ground-glass plate, sufficiently large to form a background for all the flotation vessels may be placed immediately behind the thermostat, where it can be

illuminated in a manner which will not heat the bath. This requires that the back of the thermostat also be made of glass. Local heating must be avoided at all costs. Hence, if it is necessary to have strong illumination on the divers, as is the case when observations are to be made of organisms placed in the diver for study, a special arrangement for submerging the light source in the thermostat will probably have to be made. For this purpose Holter employed lamps placed in a trough made of ground-glass plates cemented together. The trough was lowered into the thermostat and filled with water to the water level of the thermostat. The water in the trough was cooled by a cooling coil to keep the temperature a few tenths of one degree below that of the thermostat.

Fig. 112. Arrangement of Cartesian diver apparatus.
S, Syringe to vary pressure
M_M, mercury manometer
M_W, water manometer
B, air bottle
From Rocher (1943)

Convection currents in the flotation vessel, which may result from local heating, can be detected by pipetting a colored solution of the same density as that of the medium into the flotation vessel to form a colored layer in the bottom third. Any convection movement will produce colored streaks in the medium.

(2) FLOTATION VESSELS

A flotation vessel and its clamp are illustrated in Figure 113. A standard-taper ground-glass joint is used to connect the vessel through rubber pressure tubing to the manifold which leads to the manometer. All parts not surrounded by thermostat water, here as elsewhere in the apparatus, must be made small and thick-walled to reduce the air space. The angle of the conical bottom of the vessel should not be too small, since it is well to reduce the surface of contact between the end of the diver tail and the bottom of the vessel to a minimum. Otherwise there is a tendency for the tail to stick to the bottom.

The distance between the top of the diver, when the diver is at the bottom of the vessel, and the circular mark (*H*) on the vessel should be only a few mm. in order to minimize the movement of the neck seals. The hydrostatic pressure on the diver should be kept small; accordingly, the level of the medium should not be higher than *G* in Figure 113.

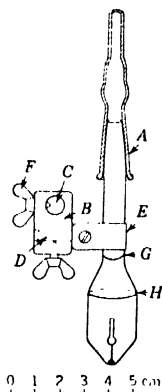



Fig. 113. Flotation vessel.
A, ground-glass joint
B, brass block
C, supporting rod (in cross section)
D, brass rod
E, brass cuff
F, wing screws
G, surface of flotation medium
H, circular mark.
From Holter (1943)

The flotation vessels are mounted by means of *B* (Fig. 113) on a brass rod running the length of the thermostat.

Observations of the diver may be facilitated by using a magnifying glass or a low-power horizontal microscope mounted so that it can be moved sidewise on a rail fastened to the outside of the thermostat. With this arrangement the microscope can be shifted for use with divers in the different flotation vessels. If the eyepiece has a horizontal cross hair, this can serve to establish the equilibrium position of the diver; in this case the circular marks on the vessels are superfluous.

(3) THE FLOTATION MEDIUM

The flotation medium originally used by the Carlsberg Laboratory workers was an almost saturated solution of ammonium sulfate, and the Cambridge group employed an 11 *N* solution of lithium chloride.

An investigation of various solutions which might be used as media led Holter (1943) to the choice of a sodium nitrate-sodium chloride solution. As Holter pointed out, a proper medium should have low viscosity, chemical stability, transparency, biological innocuity, glass-wetting properties, low and reproducible surface tension, and very little gas solubility. Holter's medium has proved satisfactory over a long period of use.

Preparation of Holter Medium. Dissolve 27.2 g. sodium nitrate, 13.7 g. sodium chloride, and 0.2 g sodium taurocholate in 59.1 ml. distilled water containing 3 drops of 0.1 *N* hydrochloric acid. To a sample of the soln. add bromocresol purple and then 0.01 *N* hydrochloric acid to bring the color to that of the indicator at *p*H 5.8 to 6.0 in salt-free soln. From the quantity of acid required, calculate the amount of 0.1 *N* hydrochloric acid to add to the main portion of the soln. to bring it to the same *p*H. Add the calculated amount and filter the medium through lintless paper, refiltering the first part of the filtrate. Any cloudiness appearing upon the addition of the acid is due to precipitation of taurocholic acid and should soon disappear. Check the density of the medium at the proper temperature with an accuracy of 0.1%; a Mohr-Westphal balance may be used.

When the gas in the diver is air and no glass stoppers are used in the neck seals to inhibit diffusion (see page 368) the medium should be saturated with air as described on page 374. When other gases are used in the diver, the flotation medium need not be saturated with these gases but the diffusion losses may be impeded by mouth seals and glass stoppers.

Dust is apt to contaminate the flotation medium after some time; hence, it is occasionally necessary to combine the contents of the vessels, refilter the solution, check the density, and refill the vessels with a pipette. The pipette is used to avoid getting the soln. on the neck of the vessel where it might crystallize.

(4) THE CONNECTING MANIFOLD

The flotation vessels are connected to the manometer by means of a thick-walled glass manifold tube (*G*, Fig. 111), carrying about

eight side tubes which communicate with the flotation vessels through pieces of rubber pressure tubing about 15 cm. long, and the standard-taper joints (*K*), which are slightly greased with vaseline. Each of the side tubes (5 cm. long and 5 cm. apart) has a glass stopcock to enable one flotation vessel at a time to be connected to the manometer. The three-way tap (*H*) enables either the flotation vessels or the manometer to be connected to the air.

(5) THE MANOMETER

The glass manometer tube has an inside diameter of about 2 mm.; each arm is around 150 cm. long. The manometer fluid is adjusted by means of the pressure regulator illustrated in Figure 114. This pressure regulator has been found to be more practical than the syringes originally used, chiefly because the latter tend to leak and also give rise to air bubbles in the manometer tube. The pressure regulator is made of a piece of rubber tubing, 30 cm. long, 2 mm. in wall thickness, and 11 mm. in inside diameter, which lies in a metal trough (*F*). The pressure screws are held by bridges (*K*) fastened to the trough. The metal cylinder (*D*) used for coarse adjustment is 15 cm. long and the width of the trough. The pitch of screw *B* causes a 1 mm. motion of *D* for each half-turn, corresponding to a pressure change of about 25 cm. in the manometer. The pitch of the fine screw (*C*) is about half that of *B* and a half-turn results in a pressure change of about 1 cm. The ends of the tubing (*A*) are closed with rubber stoppers. The reservoir (*G*) has a 20 ml. capacity. *L* and *M* are three-way taps, *H* and *J* stopcocks, and *E* a metal shoe.

Manometer Fluid. Brodie solution is used as the manometer fluid. It consists of 23 g. sodium chloride and 5 g. sodium taurocholate dissolved in 500 ml. distilled water. Dyes may be used to color the solution if desired, and it is well to store the solution over a few crystals of thymol.

The manometer is filled by drawing the solution up through *J* into *G*. The levels in the arms are then adjusted so that both menisci are about at eye level between the screws *B* and *C*.

Air Bottle. In order to render the measurement of the equilibrium pressure independent of barometric changes and, to a large degree, of temperature changes in the thermostat, one end of the manometer is connected by thick-walled capillary tubing to an air

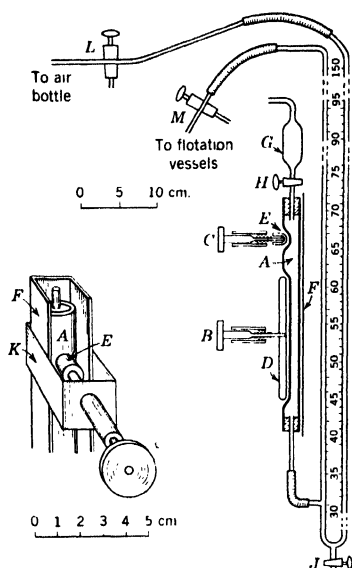



Fig. 114. Manometer and pressure regulator. At left: detail of pressure regulator. *From Holter (1943)*

Fig. 115. Cartesian diver assembly in thermostat. *From Holter (1943)*

bottle completely submerged and held to the bottom of the thermostat by lead weights (Fig. 115). The volume of the air bottle should be as large as possible (2–4 l.).

(6) THE MICROLITER DIVER

Dimensions of the Diver. The size and shape of different types of diver are shown in Figure 116. The "standard" divers are most generally employed; they have the dimensions:

Inside neck diameter	0.8 to 0.9 mm
Neck wall thickness	0.1 mm
Neck length	8–10 mm
Bulb diameter	2-3 mm.
Tail length	5–8 mm.
Total volume	10–12 μl.
Neck volume	about 5 μl.
Total weight	20–30 mg.

These dimensions have been found to be preferable to those previously used, chiefly because the longer and narrower neck enables reduction in the diffusion of gas through the seals. Regardless of how large the divers are made, the inside neck diameter should not exceed 1–1.5 mm., although seals can be placed conveniently in necks up to 2 mm. On the other hand, the diameter should not be less than 0.6 mm., because with smaller diameters the difficulty of placing neck seals is increased, due to the greater surface tension. It may not be possible to go down to 0.6 mm. in some cases because the pipettes which must be used may be too large to enter the neck. Usually the smallest neck widths have to be 0.65–0.75 mm.

In order to determine the minimum neck length, allow 1.5 mm. between the seals, which, with their menisci, require about 1.5 mm. each. The lowest seal must be about 1 mm. from the lower end of the neck.

Cylindrical divers (*C*, Fig. 116) are capable of the greatest reduction in size (down to a total vol. of about 3μl.) and the conical divers (*D*) furnish the greatest accuracy by a combination of a relatively small gas volume with a reaction mixture having a relatively large surface. The standard (*A*) and long-necked (*B*) divers are also shown in the figure.

Making the Diver. Divers may be made according to the method of Boell, Needham, and Rogers (1939) or as described by Holter (1943). The technique of the former may be applied to

relatively thick-walled divers having a total volume >10 μl., although this method involves a lack of economy of the carefully selected and calibrated capillary glass tubing from which the divers are made. Holter's method is more economical and it is far superior for divers having a total volume <10 μl.

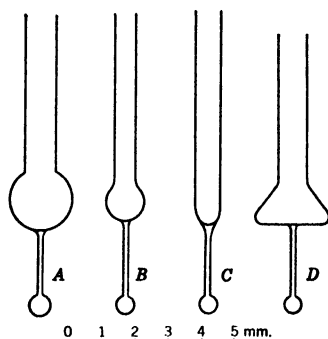



Fig. 116. Different types of divers.
From Holter (1943)

The following table has been given by Holter (1943, page 420) to serve as a guide for the selection of glass capillaries suitable for making divers:

Inside diameter of capillary (width of diver neck), mm.	Wall thickness, mm.
0.65 to 0.75	0.08 to 0.09
0.75 to 0.85	0.09 to 0.10
0.85 to 1.0	0.10 to 0.12
>1.0	0.12 to 0.15

The capillaries should be made from a stock of glass tubing having a ratio of 1:10 between the inside diameter and the wall thickness. The density of the glass should be measured, since this value enters into the calibration of the divers. The waste glass left after drawing the capillaries should be saved and used for making the diver tails. Entrapped air bubbles, which may be visible only as streaks, sometimes occur in the glass tubing. It is important that the glass used for divers has no entrapped air, which would change the density of the diver; accordingly, the glass should be tested by fusing one end of the capillary into a ball and examining for air bubbles.

To make a diver by the method of Boell et al.:

1. Fuse one end of a suitable length of capillary tubing and draw out a solid tail as indicated in steps *A–C*, Figure 117. The tail must be light enough not to lose its alignment when the bulb is being blown.

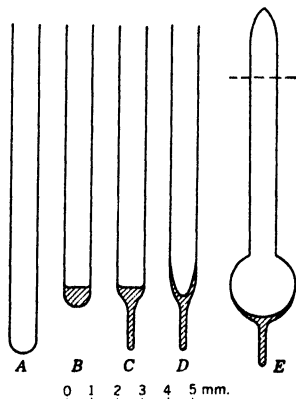



Fig. 117. Making of diver according to Boell, Needham, and Rogers. *From Holter (1943)*

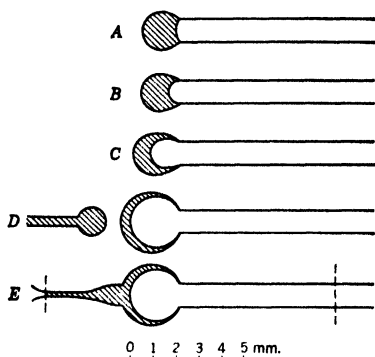



Fig. 118. Making of diver according to Holter. *From Holter (1943)*

2. Thicken the walls above the tail (step *D*, Fig. 117).
3. Seal the open end and heat carefully over a micro flame while rotating the tube so that a bulb will blow itself (step *E*, Fig. 117). The size of the bulb will be determined by the vol. and temperature of the air in the sealed unit.
4. Cut off the neck at the length desired.

To make a diver by the method of Holter:

1. Connect a long thin-walled piece of rubber tubing to one end of the capillary chosen for the proper inside and outside diameters.
2. Fuse the other end of the capillary in a micro flame to form a glass drop (*A*, Fig. 118) or, to economize on capillaries, use waste glass to form a drop and then seal it to the end of the capillary. Take care that no air bubbles are entrapped in the glass. The size of the drop will determine the size of the bulb.
3. Rotate the capillary and hold it horizontally while heating the drop in the flame. Carefully blow a bulb, moving the end out of the flame so that the heat is applied only to the thick-walled portion of the growing bulb (*B, C, D*, Fig. 118).

4. Fuse the end of a glass thread to form a drop, and fuse the drop to the bottom of the bulb by heating the drop to a bright red heat and the very bottom of the bulb to a medium red heat, and then joining the two with a slight pressure.

5. Remove from the flame and pull out the glass tail at once (*E*, Fig. 118). If the bulb is too hot, its shape will be spoiled while pulling the tail; if too cold, the glass drop will not fuse into the bottom and the tail will break off. Test the strength of the fusion by applying a lateral pressure to the tail.

6. Cut off the excess at the neck and tail with a diamond point.

To prepare a conical diver (*D*, Fig. 116) heat only the side walls of the bulb after the tail has been fused on, and collapse and draw into the conical shape.

Cleaning the Diver. Holter (1943, page 429) has recommended the following procedure for cleaning the diver after an experiment:

1. Rinse the outside of the diver with distilled water.

2. Remove the neck seals separately with soft filter paper cut into short narrow strips and rolled between the fingers to form tight smooth rolls. Take particular care to remove the paraffin oil as completely as possible.

3. With a finely drawn pipette having a 2–3 ml. capacity, fill the diver with glass-distilled water and blow about 2 ml. water through the diver in a brisk stream. Stop before air gets into the diver so that filling with the next liquid will be easier.

4. Repeat the washing with acetone, toluol, acetone, and twice with glass-distilled water in the order given. Leave the toluol in the diver for a few min. before replacing it with acetone. If the diver had a neck ring of wax, rinse two to three times with toluol.

If the diver is unusually dirty, treat as described and then fill it with freshly prepared 1% potassium permanganate in conc. sulfuric acid, and let stand overnight. Replace the solution with water, wash with strong hydrochloric acid, and flush well with glass-distilled water.

5. Blow air through the diver to remove as much water as possible after the final washing.

6. Dry the outside of the diver with filter paper and place in an oven at 120°.

7. Holding the diver by the tail with forceps, heat only the neck of the diver by moving it slowly two to three times through

a micro flame. The flame should acquire a faint sodium tinge.

This process can be replaced by heating for about 20 min. at 400° in a furnace. It is necessary to heat the diver neck to insure proper moistening of the neck by the seals.

8. Handle the clean divers only with clean forceps (cork-tipped forceps are recommended). Clean divers should never be touched with the fingers. Store the divers in stoppered glass tubes.

Adjusting the Weight of the Diver. The weight of the diver must be adjusted so that when it is finally charged for an experiment the equilibrium pressure will approximate the barometric pressure. The desired weight of the diver may be calculated from the formula:

$$g_D = \frac{V_t\phi_m - V_{oil}\phi_{oil} - V_w\phi_w - V_m\phi_m}{1 - (\phi_m/\phi_{gl})}$$

where g_D is the desired weight of the diver, V_w the aqueous volume in the bottom and neck of the charged diver, V_t the total diver volume, V_{oil} the vol. of the paraffin oil seal, V_m the vol. of the mouth seal, ϕ_m, ϕ_{oil}, ϕ_w, ϕ_{gl} the densities of the medium, paraffin oil, aqueous charge, and glass, respectively. The total volume (V_t) is measured by weighing the diver to 0.1 mg., first empty, and then filled with water. The densities of the liquids may be determined with a Mohr-Westphal balance, and the glass density by weighing 2–5 g. of the glass in air and in water to 1 mg. If air bubbles are present in the glass of the diver, the density can be obtained by the flotation method. Holter has given the following proportions of ethylene dibromide and bromoform to make flotation liquids of the densities indicated:

Ethylene dibromide, ml.	Bromoform, ml.	$d_{40}^{22.50}$
20	5	2.314
15	10	2.460
15	15	2.529
10	20	2.643

Of course it is possible that different grades of the two compounds will give mixtures with different densities. The following example of the calculation of the desired weight of a diver has been taken from Holter (1943, page 427): "A diver made from Jena glass of specific gravity 2.40 weighs 12.9 mg. empty and 23.6 mg. filled with water.

It is to be used for the determination of the respiration intensity of infusoria and is to contain the following charge: at bottom: 0.8 μl. of the infusoria culture medium; in the neck: 0.7 μl. $N/10$ NaOH, 0.7 μl. paraffin oil, and a mouth seal 2 mm. long. The inside diameter of the diver's neck is 0.75 mm., its area 0.445 mm.^{2}, hence the volume of the mouth seal 0.89 μl. ϕ_w, the density of the bottom drop and the $N/10$ NaOH is practically $= 1$, $\phi_{oil} = 0.87$, $\phi_m = 1.325$, $V_t = 23.6-12.9 = 10.7$ μl.

$$g_D = \frac{10.7 \times 1.325 - 0.7 \times 0.87 - 1.5 \times 1 - 0.89 \times 1.325}{1 - (1.325/2.40)}$$

$$\frac{10.89}{0.448} = 24.3 \text{ mg.''}$$

The weight of the diver may be adjusted by cutting off part of the tail or fusing more glass onto it. If too heavy, a little more of the tail is cut off than necessary and the diver is brought to the proper weight, within 0.1–0.2 mg., by fusing on measured lengths of glass thread of known diameter. Finally, the threads are fused into a ball at the end of the tail, and the diver is dried and weighed to 0.05 mg.

The weight of the diver is checked by charging it with the quantities of the liquids chosen in the calculation, and determining its equilibrium pressure. If properly prepared, the equilibrium pressure will not deviate more than 20 cm. from the barometric pressure.

Devices for Holding the Diver. The diver is charged by raising it on a mechanical stand so that the tip of the pipette, which is stationary, can be brought exactly to the position desired and the contents of the pipette discharged at the proper place in the diver. Special diver holders have been described by Holter which facilitate the pipetting manipulations.

For divers with short necks ($\gtreqless$ 1 mm. in width) the simple rubber tubing holder, shown in Figure 119, may be used. For divers with narrow necks, the centering with respect to the stem of the pipette is more difficult, and, accordingly, Holter devised a clamp stand which can be fixed relative to the pipette so that repeated centering is not required. The clamp stand is shown in Figures 120

and 121. The tongue on the left in Figure 121 moves on a horizontal axis and presses the neck of the diver into a vertical V-shaped groove in the head of the stationary post on the right. The edge of the tongue is curved so that it touches the diver only at one point. The tension needed to hold the tongue against the diver is derived from a light spring which presses against the handle of the tongue

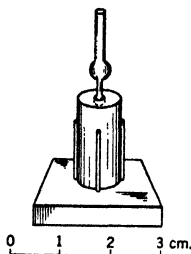



Fig. 119. Diver support made of pressure tubing.
From Holter (1943)

(Fig. 120). The entire clamp is fitted on the top of a rack and pinion stand, which enables the diver to be raised or lowered evenly. The base of the rack and pinion stand has leveling screws so that the inclination of the diver can be controlled. A mirror fitted to the side of the stand enables the operator to observe the centering of the pipette from the side, and a magnifying glass attached to the front of the stand or used in a head band permits the operator to observe more easily from the front.

The diver is placed in the flotation vessel and removed from it by means of a loop at the end of a piece of stainless steel wire about 15 cm. long. The loop is made a little smaller than the diver bulb so that the diver can rest on the loop with its tail hanging down through the hole, and the loop is bent at right angles to the wire so that the diver is upright when the wire is held vertically.

(7) THE PIPETTES

The pipettes used with the divers are straight glass capillaries designed to measure 0.2–2 μl. volumes with an accuracy of 1%. They are filled and emptied by mouth through a piece of rubber tubing. Four types of pipettes are used with microliter divers.

Type 1 Pipette. This form of pipette (Fig. 122) is intended for measuring dilute aqueous solutions, particularly for neck seals. The pipettes are drawn from thermometer tubing having a bore of 0.2–0.3 mm. The tubing is heated and the capillary widened into a bulb

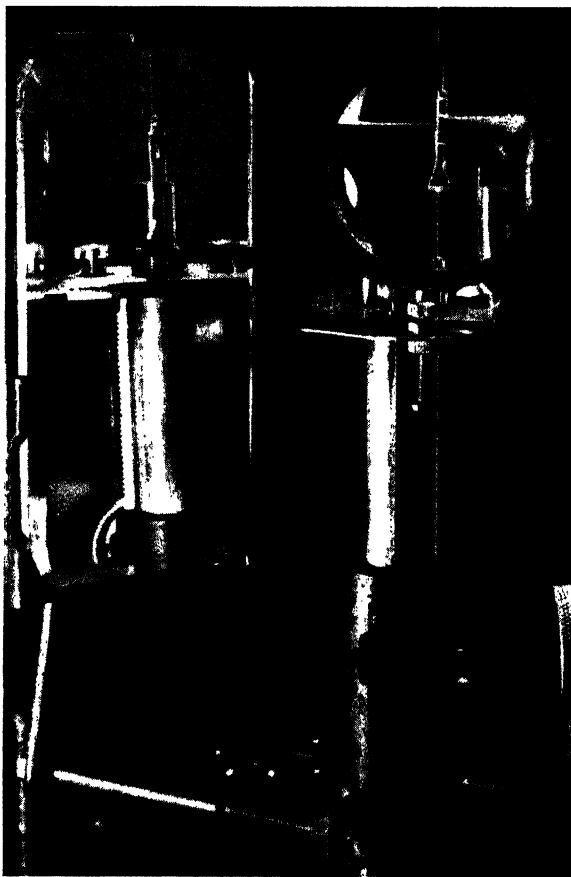



Fig. 120. Clamp stand for divers. *From Holter (1943)*

having a wall thickness about half the diameter of the bulb. A slight pull is applied to give the shape in *A*, Figure 122. At the point indicated by the arrow, heat is applied with a micro flame and the tubing is pulled out to give the pipette (*B*, Fig. 122). The

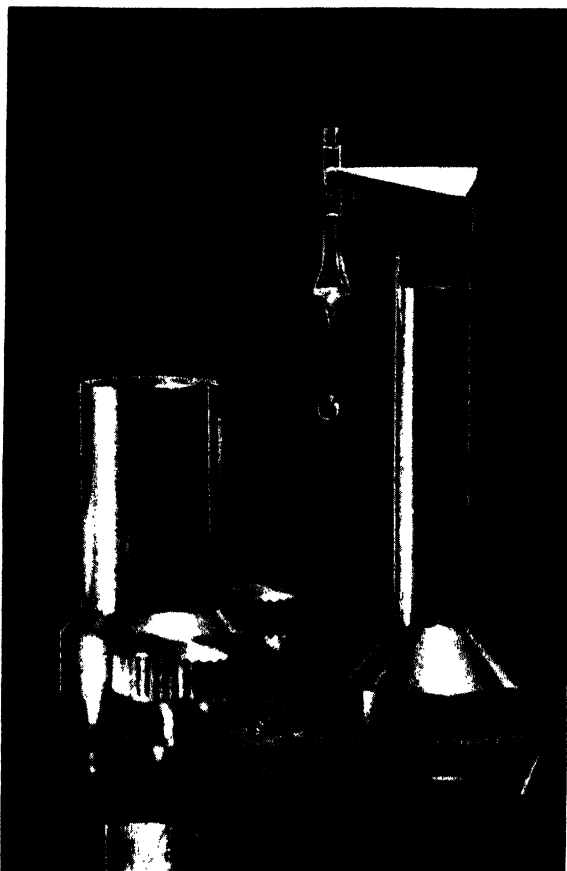



Fig. 121. Detail of clamp stand.
From Holter (1943)

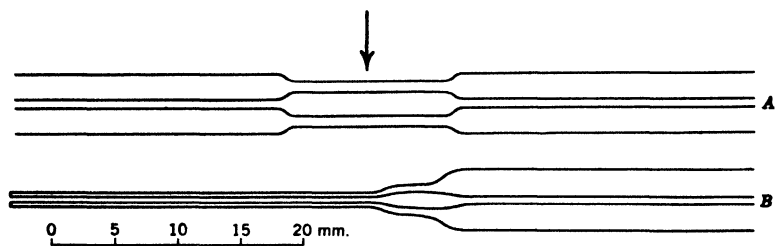



Fig. 122. Making of type 1 pipette, two successive stages.
From Holter (1943)

inside diameter of the tip of the pipette should be about 0.07–0.12 mm., and the outside diameter about 0.15–0.25 mm., depending on the size of the diver employed. The delivery end of the pipette should be strictly cylindrical for about 1 cm. from the end, or, even better, slightly trumpet shaped at the tip. This form minimizes the tendency of the liquid to creep up on the outside.

Type 2 Pipette. This pipette resembles the type 1; however, it is intended for viscous liquids, particularly for paraffin oil. The orifice at the tip must have a diameter no less than 0.15–0.18 mm. and the glass wall thickness must be about 0.04 mm. In making the pipette, the ratio of wall thickness to diameter of the bulb (*A*, Fig. 122) should be about 1:4, and the pipette is drawn to a tip with an outside diameter of 0.2–0.25 mm. Paraffin oil has no tendency to creep so that the exact shape of the tip is not important.

Type 3 Pipette.* This form of pipette is known as a "braking pipette," and is intended for the transfer of cells and pieces of tissue together with known amounts of liquid. The pipette (Fig. 123) is drawn out at its upper end to a hair-thin tip so that the rate of filling and emptying will be determined by the rate at which air can pass through the very fine tube or "brake" (*E*). The outside diameter of the pipette (*A*, Fig. 123) is 0.3–0.5 mm. The cork (*B*) is smoothly cut in half lengthwise and a fine longitudinal groove is made in the cut surface to hold the pipette. The rubber tubing (*D*) is used for the operation of the pipette by mouth. *C* in the figure indicates the jacket tube.

In preparing the pipette, the upper end is drawn out long and with a gradual tapering. Small portions are broken off with a fine forceps until the rate of filling and discharge of the pipette is suitable. To test this, dip the mouth of the pipette into water and observe the rate at which the water rises by capillarity. A rate of about 1–5 mm./sec. has been found to be about right. The dimensions of the delivery end of the pipette will be determined primarily by the size of the object to be transferred. Whenever possible, taper the mouth of the pipette, since it is difficult to reproduce the size of the last droplet remaining in the mouth after delivery.

When in operation, should the braking tip of the pipette become filled with condensed water which cannot be dislodged by sucking or blowing, apply a slight excess pressure to the jacket tube and

* See Bibliography Appendix, Ref. 47.

close off the rubber tubing with a pinchcock. Heat the jacket tube cautiously with a gas flame in the region of the braking tip; this will cause the water in the tip to evaporate and the warm air will clear the fine channel. If the tip becomes filled with a salt solution, it will usually be necessary to draw a new tip. Obviously, it is desirable that the pipette be rather long when first made.

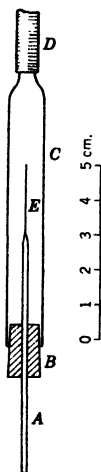
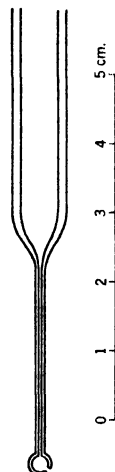
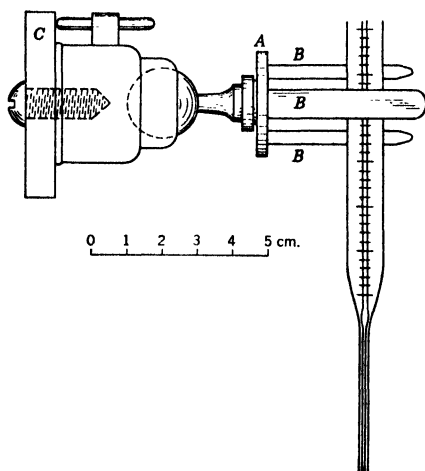



Fig. 123. Fig. 124. Fig. 125.

Fig. 123. Braking pipette. *From Holter (1943)*
Fig. 124. Ball-tipped pipette. *From Holter (1943)*
Fig. 125. Ball joint for holding pipettes. *A,* Supporting discs; *B,* metal springs; *C,* supporting rail. *From Holter (1943)*

Type 4 Pipette. The ball-tipped pipette (Fig. 124) is used to deposit a drop of liquid at a definite place in a very narrow diver neck ($<$0.7 mm. diameter) or in a fine capillary. The stem attached to the ball has an outer diameter of 0.15–0.25 mm. and the ball itself a diameter of 0.2–0.4 mm.

The pipette is made by drawing out a glass capillary from a piece of tubing of medium wall thickness, sealing the tip by fusing, and connecting the pipette with rubber pressure tubing to a source of compressed air at 0.5 to 2 atmospheres (depending on the size of the ball to be made). The sealed tip is brought close to a micro flame; when the glass becomes soft enough it blows out into a ball which perforates at the softest point.

Devices for Holding Pipettes. In order to allow convenient adjustment of pipettes, clamps fitted to universal ball joints were used by Holter (Fig. 125). The clamps were mounted on a horizontal bar, under which a ground-glass plate was fixed and illuminated from behind to furnish favorable light for the pipetting. Pipettes which must be removed from their clamps to obtain the sample, or for any other reason, should be marked so that they can be re-clamped at exactly the same place in order to avoid the necessity of recentering them with respect to the diver neck when the diver is held in its clamp stand. When it is desirable to use several pipettes in quick succession with the same diver, the pipettes must all be aligned to the diver, and the diver stand must rest on a surface smooth enough to enable it to be pushed under the various pipettes without losing the alignment. A level glass plate on the top of the table is recommended.

Calibration of Pipettes. Pipettes of types 1 and 3 are calibrated by iodometric (1 *N* potassium iodate) or acidimetric (1 *N* potassium hydroxide) microtitration of a pipetted vol. of liquid using 0.01–0.02 *N* standard solutions for the titration. Type 2 pipettes are calibrated by weighing paraffin oil delivered from the pipette directly on to the pan of a microbalance.

Calibration markings on types 1, 2, and 4 pipettes are placed far up on the pipette in the form of strips of millimeter paper. If the pipettes are made from a broken thermometer, the graduations on the glass will serve. A thin-walled and expanded portion of the pipette, 5–10 mm. long, should lie between the fine stem and the graduated part (Fig. 125). Type 3 pipettes require only one mark, since they are used for complete discharge. No very satisfactory means has been found for placing the mark when it must be so near the end as to enter the diver neck during pipetting. Loops of hair, held by a tiny bit of picein or glass cement, are usually too bulky. Glass ink covered with lacquer is soon worn off. The Carlsberg Laboratory group prefer no markings at all, but, after the pipette has been filled according to judgment under a binocular microscope, the length of the liquid column in the pipette is measured to 0.1 mm. by means of a micrometer scale. The pipette is emptied into the bottom of the diver, and if the volume was misjudged, the difference is made up by correspondingly changing the volume of one of the neck seals or the mouth seal. When the pipette

is narrow, the calibration mark will come high enough on the stem to avoid contact with the diver, and in this case a hair loop or a glass ink mark may be used.

(8) FILLING THE DIVER

The Bottom Drop. According to the calculations of Linderstrøm-Lang (1943), the general rule follows that the thickness of the layer of the bottom drop of reaction mixture must not exceed 0.5 mm. if the rate of gas diffusion through the drop is not to become the limiting factor for the rate of the gas exchange to be measured. This refers to gas changes in the range of those to be expected in the usual biological systems (of the order of 0.01 μl./hr.). For all other cases, the equations given by Linderstrøm-Lang (1943) should be applied to check the conditions.

It is essential that the bottom drop spread to reduce the thickness of the liquid layer. The cleanliness of the diver is an important factor. If the reaction mixture does not wet the glass, recourse may be had to one of the following devices:

1. Addition of a surface-active substance, such as 0.1% sodium taurocholate, provided that the substance will not interfere with the reaction.

2. A very short centrifuging, or whirling by the arm while holding the diver (the diver must be held with cloth or filter paper to prevent the fingers from touching it).

3. If centrifuging is not sufficient, and it is permissible in the experiment, moistening the wall of the diver bulb before the bottom drop is added by filling the bulb to within 1 mm. of the neck with a suitable liquid and then sucking it out as completely as possible. It will be necessary to correct the volume of the bottom drop for any of the liquid not removed. When the moistened part is less than 2 mm. from the lowest neck seal there is danger from creeping; this may be obviated by a wax ring, described on page 363.

In order to avoid injuring cells, or damaging the tip of the braking pipette used to transfer them by having the tip touch the glass bottom of the diver, a small amount of a suitable liquid (about 0.1 μl.), termed a "forerunner" by Holter, is placed into the bottom of the diver first. The "forerunner" is introduced with a slender flexible pipette which will not break if it touches the bottom of the diver. It is necessary to avoid evaporation of the "forerunner" by

keeping the diver in a moist chamber until time for adding the main bottom drop.

Should the biological object be so large that it cannot be transferred into the diver by means of a pipette, the diver is filled completely with the bottom drop solution, the diver is lowered into a dish containing the object, and the object is led into the mouth of the diver. Then the diver is placed upright and the object sinks to the bottom. The excess solution is pipetted out, the outside of the diver is dried and small rolls of lintless filter paper are used to dry the inside of the diver neck, and finally the weight of the remaining liquid plus the object is determined after a neck seal of paraffin oil has been placed to prevent evaporation.

To improve the visualization of the object in the bottom drop during the pipetting, a drop of water is used to make a small piece of cover slip adhere to the diver bulb after the diver has been placed in the clamp stand. When the diver is in the thermostat, the best view of the object may be had by using a cylindrical diver or placing the object in a neck seal if this is possible.

In order to remove an object from a diver without injury, the neck of the diver is first rinsed as described on pages 364–365; then the whole diver is filled with a suitable liquid, the diver is placed in the dish intended for the object, and the object is allowed to float or sink out of the diver. A slender pipette filled with the liquid may be used to flush the object out of the diver.

Wax Rings. There is great danger that the bottom drop will creep up and be drawn into the capillary space between the stem of the pipette and the diver neck when very small divers are used. Proper centering of the pipette may not be sufficient to avoid the difficulty. In such a case, a ring of beeswax about 0.5 mm. wide is placed at the base of the diver neck. This will also prevent the neck seal from creeping down.

To place the wax ring an electrically heated platinum loop (Fig. 126) is used. When the diameter of the neck is greater than 1 mm., carry out the following operation by hand:

1. Heat the loop and fill it with wax.
2. Allow to cool and place in the diver neck.
3. Apply the ring where desired by heating to melt the wax and rotating the loop to form a ring.
4. Move the loop away from the glass wall and let it cool.

5. Remove the loop from the neck.

For divers with smaller necks, the loop apparatus is clamped horizontally, the diver is held horizontally in a diver clamp, and the wax ring is placed by rotating the diver smoothly about its long axis. A dissecting microscope assists in the observation and control of the operation.

Neck Seals. Neck seals are placed by bringing the end of the pipette (type 1 or 2) into the neck so that the pipette stem is carefully centered. Then, by blowing cautiously a free-hanging drop is formed which slowly grows (*A*, Fig. 127) until it touches the neck wall (*B*) and finally forms the seal (*C*). If the centering is inexact the liquid may spread between the pipette and the neck wall.

With wide necks it may be necessary to add more liquid to form the seal than is desired in the final seal. Should this be the case, the tip of the pipette is raised from the bottom meniscus to the interior of the seal, and the excess liquid is drawn up into the pipette. Hence, pipettes used for neck seals must either have two marks, the volume between them being that desired in the final seal, or they must have calibrated cylindrical bores such as are obtained when the pipettes are made from broken thermometers.

When coarse-tipped pipettes are employed or the divers have very narrow necks, care must be exercised in removing the pipette from the seal to prevent pulling up the liquid. The pipette should be drawn out of the seal with a jerk. Similarly, when the ball-tipped pipette is used, the ball should be removed from the drop deposited by carefully moving the ball until it is just underneath the surface of the drop, and then pulling it out with a jerk.

When it is necessary to exchange neck seals without disturbing the bottom drop, the seals are first carefully removed with small rolls of filter paper, soft paper rolls being used to absorb most of the liquid; hard lintless paper rolls which almost fill the neck are used to effect the final cleaning and drying. The neck is then filled with water by bringing the tip of the pipette to a point about 1 mm. below the bottom neck seal and blowing out the water very carefully. Once the neck is filled. the cautious blowing is continued to rinse out the neck with a slow stream of water. Finally the water is removed from the neck and filter paper is used for drying. There is danger of evaporation of the bottom drop as long as there is no oil seal present, therefore the exchange of seals must take place

as rapidly as possible. When it is especially important to guard against evaporation of the bottom drop, the operations should be performed in a moist chamber.

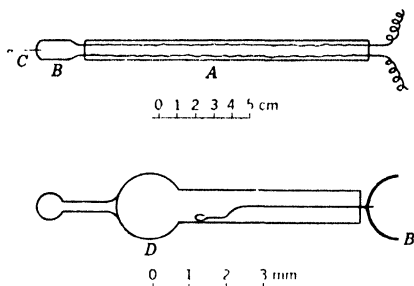



Fig. 126. Platinum loops for placing wax rings. Above, general appearance; below, detail. *A*, glass tube; *B*, 0.5 mm. platinum wire; *C*, loop of 0.2 mm. platinum wire; *d*, diver. *B* and *C* are welded together. Current needed is 2–6 amp. *From Holter (1943)*

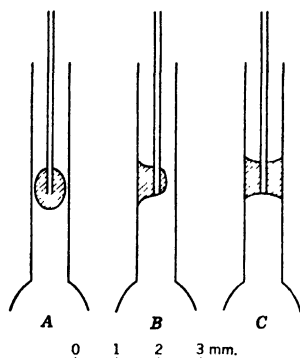



Fig. 127. Formation of neck seal; three successive stages. *From Holter (1943)*

In general, the neck seals should not be less than 0.5 mm. in length, *i.e.*, the distance between the apexes of the opposing menisci, and there should be at least 1 mm. of dry glass between the seals to prevent their mixing. Oil seals should be about 0.5 mm. long and other seals about 1 mm. The distance between the oil seal and the mouth seal should always be as small as possible in order to keep the volume of the air space between them as small a fraction of the total gas volume as possible (Linderstrøm-Lang, 1943).

Aqueous Neck Seals. To insure proper wetting of the neck wall by an aqueous seal, it should contain 0.1% sodium taurocholate if the compound can be used in the experiment. The aqueous neck seal should also be isotonic with the bottom drop to avoid distillation. In respiration work a seal of 0.1 *N* sodium hydroxide may be used, since the difference in vapor tension between this solution and water is small enough to be unimportant.

Should liquid be drawn along the diver neck accidentally, when placing the aqueous seal, and if it is not feasible to repeat the filling, the moistened area may be dried with filter paper. It is particularly important that no moisture be present on the neck above the oil seal since the water would distill into the mouth seal during the experiment.

The Paraffin Oil Seal. Highly refined colorless paraffin oil having a viscosity of about 20 Engler degrees at 20° and a density of about 0.87 will suffice for most purposes. Of many liquids tested, this has been found to be the best for sealing off the reacting system from the outside.

Seals Containing Living Organisms. In order to place a living object in a seal without damage, it is first necessary to place a seal of the aqueous medium in the neck and then introduce the object into the seal with a braking pipette.

The Mouth Seal. The mouth seal serves to prevent the loss of gas from the diver, and it enables an adjustment of the equilibrium pressure of the diver to a chosen value. In general, the length of the seal must be several mm. to prevent significant gas loss, and the length must be adjusted with an accuracy of 0.1 mm. to obtain an equilibrium pressure within about 5–10 cm. of the desired value. The length of the mouth seal is determined by measuring the distance between its lower meniscus and the diver mouth by means of a traveling microscope (Fig. 128). The mouth seal is made as follows:

1. After placing the oil seal, bring the tip of an "air pipette" (a braking pipette with a finely drawn delivery tip having an inside diameter of about 100 μ and an outside diameter of about 150 μ) into the diver neck to a point about 1 mm. below the position at which the lower meniscus of the mouth seal is to be placed.

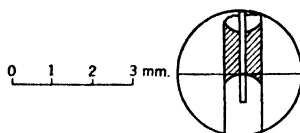
2. Adjust the measuring microscope until the horizontal cross hair in the eyepiece coincides with the top point on the rim of the diver's mouth. Then lower the microscope by means of the micrometer screw until the cross hair comes to the point where the bottom meniscus of the seal is to be.

3. Place a seal of diluted flotation medium around the stem of the braking pipette at the mouth of the diver with a fine hand pipette. (The diluted medium is employed to avoid crystallization in the mouth seal. It is prepared by diluting the ordinary medium with an equal volume of water and adding sodium taurocholate to 0.1 to 0.2 % to insure that the menisci will be properly formed.)

4. Apply suction to the braking pipette to draw the seal down until the bottom meniscus reaches a position about 0.5 mm. below the microscope cross hair; then raise the seal by blowing until the

meniscus comes to rest at the position of the microscope cross hair. The appearance of the seal at this stage should be that shown in Figure 128. (By drawing the seal below the final position, the glass walls will be moistened and the lower meniscus will not be distorted. The upper meniscus should also be well developed and for this reason the seal should not reach the rim of the neck. When the two menisci equally oppose one another's surface tension, the mouth seal will stay in place.)

5. Lower the diver stand quickly to remove the "air pipette" from the diver, and fill up the mouth seal to the brim. Immediately remove the solution which partly fills the air pipette when it is taken out of the seal, and rinse with distilled water to prevent clogging.

Fig. 128. Placing of mouth seal. *From Holter (1943)*

When the diver is submerged, the diluted medium in the seal is displaced by the undiluted medium in the flotation vessel. This displacement will be complete in a few min. with mouth seals 1–1.5 mm. long and 1 mm. in diameter. Should it be necessary to establish the initial equilibrium pressure more quickly, and if the mouth seal must be long and narrow, the equalization of the concentration of the liquid in the seal must be speeded by displacing the liquid with the flotation medium by rinsing. The rinsing process is carried out by first drawing up into a glass tube, which has been drawn out to a capillary about 10 mm. long and 0.2 mm. inside diameter, about 1 ml. of medium. Then the tip of the capillary is introduced into the mouth seal and the medium in the tube is blown in to displace that in the seal.

If crystallization occurs in the mouth seal, equalization of the diver will be delayed for hours, and displacement of the liquid by rinsing may not help since crystals sometimes hide in the meniscus edges and escape the rinsing current.

The length of the mouth seal and the diver charge must be chosen to give the diver the weight required for it to have an initial equilibrium pressure of about 1 atmosphere. The weight may be

adjusted by changing the length of the mouth seal. Should it be necessary to change the volume of other liquids in the diver, the equilibrium pressure can be kept constant by changing the volume of the mouth seal according to the relationship:

1 vol. aqueous soln. (d = 1) = 0.76 vol. medium (d = 1.325)
1 vol. paraffin oil (d = 0.87) = 0.64 vol. medium (d = 1.325)

In order to replace the mouth seal with one of another vol., the diver is removed from the flotation medium, rinsed on the outside with distilled water, and dried with cloth or filter paper. Then the mouth seal is removed with a pipette or filter paper rolls, and replaced with a seal of water extending into the diver deeper than the previous seal. This water seal is flushed with fresh water by the rinsing technique (page 367). The water is removed, the mouth is dried with hard filter paper, and the new seal of medium is introduced.

The length of the mouth seal may also be corrected by diluting it with water, removing the excess with a roll of filter paper, and then adjusting the position by means of an "air pipette" in a manner similar to that employed when dealing with divers in anaerobic experiments (page 373).

Seal Stoppers. Linderstrøm-Lang and Holter (1942) pointed out that the diffusion of gas through seals can be reduced considerably by placing glass stoppers into the seal. The stopper consists of a bit of glass rod having a diameter about 50–80 μ less than that of the inside of the diver neck. The length of the stopper will depend on the magnitude of the effect desired, but in general they are used 2–3 mm. long.

The solid stopper is the easiest to make but its weight may be too great, making it more feasible to use hollow stoppers. Solid stoppers can only be used with divers having a thin-walled neck and a low center of gravity, since otherwise they render the diver top-heavy. The hollow stoppers are made under a dissecting microscope from a glass capillary of the proper outside diameter by fusing one end shut in a micro flame, cutting off the capillary 0.5 mm. longer than the stopper is to be, and fusing the open end shut. The hot sealed end begins to bulge out due to the internal pressure

of the heated air, so it is necessary to remove it from the flame before the enlargement exceeds 10–15 μ.

A stopper is placed in an oil seal by introducing it into the diver with fine forceps and dropping it into the oil seal. A bubble, usually found under the stopper, is removed and the stopper is completely submerged at the same time by pushing the stopper down with the tip of the oil pipette until the bubble bursts at the lower meniscus. To remove a stopper from an oil seal, the mouth seal is first removed, and the mouth of the diver is rinsed with water and dried with filter paper rolls. Then the whole space over the oil seal is filled with oil and the diver is inverted into oil to let the stopper fall out

A stopper is placed in the mouth seal by the following procedure:

1. Place the fully charged diver in the flotation vessel where it will remain at the surface because it has been calibrated to bear a stopper and is therefore too light.
2. Rinse the mouth seal with medium (page 367).
3. Pick up the stopper with the forceps especially designed to handle it (Fig. 129) and place the tip of the stopper which protrudes from the forceps into the mouth of the diver. Linderstrøm-Lang and Holter (1942) describe the forceps as follows:

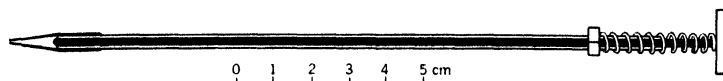



Fig. 129. Forceps for placing glass stoppers.
From Holter (1943)

"It consists of a piece of thin metal tube, 15 cm. long and 2–5 mm. outside diameter, which encloses a metal pin, 1.5 mm in diameter, 2 cm. longer than the tubing and ending in a knob. On its lower end the tube carries two narrow sheets of springy metal, 2 mm. wide and 2 cm. long, which are soldered on to the outside of the tube in such a fashion as to protrude 15. mm. over the mouth of the tube, parallel to its axis. By a slight bend the sheets are made to meet at their ends, thus forming a pair of tweezers. Between the knob at the upper end of the pin and some kind of button near the upper end of the tube lies a spring which, when at rest, keeps the pin partially lifted out of the tube. By pressing the knob of the pin down one forces the tweezers apart, thus releasing any object which they have held."

4. Slowly push the diver, with the stopper partway in its mouth, down to the bottom of the flotation vessel.

5. Release the forceps and push the stopper deeper into the mouth with the tip of the forceps. The diver will not rise if the charge has been properly calculated. However if the diver should rise, push the stopper deeper into the mouth.

Hollow stoppers must not have a specific gravity less than that of the medium or they will float out of the mouth seal. A stopper is removed from a mouth seal by inverting the diver, dipping its mouth into water, and letting the stopper fall out.

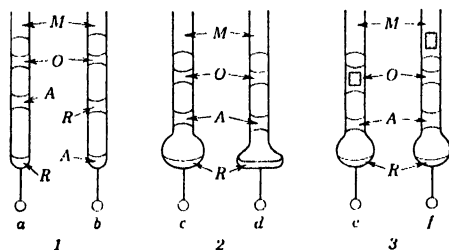



Fig. 130. Various arrangements for respiration measurements. *From Holter (1943)*

In the figure, *1* shows the cylindrical diver for use when the main requirement is small volume: *a*, gas exchange between *R* and gas phase sufficient, also when the surface of *R* is small; *b*, the gas exchange requires a large surface of *R* (only possible in case of organisms that can rest on an air meniscus). *2* shows the flask-shaped diver for use when the main requirement is a large surface of *R* and when air support is not tolerated by the organism: *c*, relatively large gas space; *d*, gas space as small as possible; the neck diameter and the length of *M* depend on the importance of preventing the gas loss by diffusion. In *3* is seen the glass stopper for use when the main requirement is that the diver be as gas-tight as possible: *e*, glass stopper in *O*; *f*, glass stopper in *M*.

The type of diver used and the arrangement of the seals will be determined by the particular experiment to be performed. The following basic set-ups (Fig. 130) were given by Holter (1943) for a simple measurement of respiration which requires only one aqueous neck seal (sodium hydroxide to absorb carbon dioxide): "Sugges-

tions regarding the choice of diver type and arrangement of seals under different experimental conditions. Assumed: A diver charge like that used in the measurement of respiration, comprising a reaction mixture (*R*) containing the organism, an absorption seal (*A*), a seal of paraffin oil (*O*), a mouth seal (*M*). The gas phase consists of air."

(9) FILLING THE DIVER UNDER ANAEROBIC CONDITIONS

In their studies on anaerobic respiration in the amphibian embryo, Boell, Needham, and Rogers (1939) employed an apparatus which permitted charging the diver in a nitrogen atmosphere. Holter (1943) described an apparatus which has the advantage of permitting small and narrow-necked divers to be filled more easily under anaerobic conditions. The apparatus of Boell *et al.* is shown in Figure 131, and that of Holter in Figure 132.

In Holter's apparatus (Fig. 132) the mercury vessel is mounted on a stand with a rack and pinion so that it can be moved up and down. *E* shows the base plate, *F* the diver clamp, and *G* the mercury vessel. The glass tube (*K*) must be wide enough to allow the diver to be moved horizontally to bring it under the various pipettes. The gas enters through tubes *C* and *D* and goes out through the bubble counter (*L*). In the arrangement shown, pipette *A* is used for an aqueous solution such as sodium hydroxide, pipette *B* for the oil seal, and *C* to introduce the gas into the diver. The latter pipette is drawn to an outside diameter of 0.3–0.5 mm. at the tip. The pipettes must be drawn so that the fine stems are precisely coaxial with the main tubes; only when the stems are parallel to one another will they all fit into the diver. The upper tubes of *A*, *B*, and *C* are heated and cemented together with picein, and, if the pipette stems are parallel, the tube *D* is joined to the group and they are all sealed air-tight with picein or wax into a wide hole in the rubber stopper. Rubber tubing is connected to the upper ends of the tubes. The liquids which are to be pipetted are placed in small tubes drawn out at the mouth to form a stem thin enough to be held by the diver clamp.

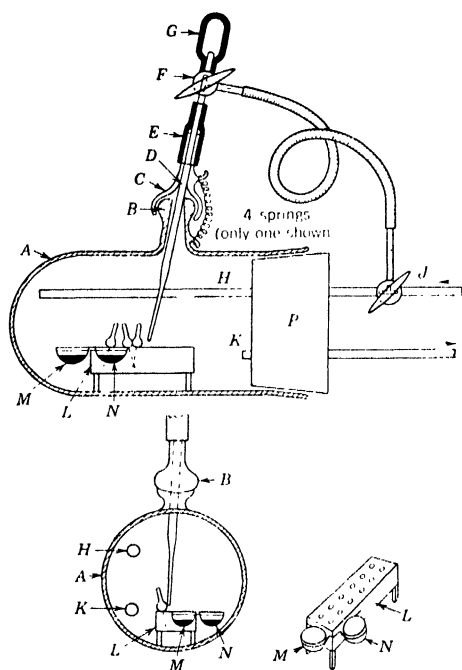



Fig. 131. Apparatus for filling divers anaerobically.
From Boell, Needham, and Rogers (1939)

Fig. 132. Apparatus for filling divers under anaerobic conditions.
From Holter (1943)

A is the filling chamber. "It bears a glass universal joint, *B*, through the upper shank of which (*C*) a tube *D* ending in an almost capillary point is fixed by means of the rubber connexion, *E*. The pipette *D* is vaselined so as to be movable vertically as well as from side to side. At its upper end it carries a three-way tap, *F*, and a teat, *G*. The whole chamber is closed by the rubber stopper, *P*, through which an entry tube (*H*) brings in the gas mixture (previously purified in the usual way by passage over metallic copper in an electrically heated furnace). The entry tube also has a three-way tap, *J*, so that part of the stream can be diverted to pass through the pipette *D*. The exit tube is represented at *K*. We found it convenient to insert a wash-bottle between the electric furnace and the entry tube *H*, so as to ensure that the entering gas mixture was not unduly dry, and also a mercury trap in case of excess pressure. Inside the filling chamber there is placed a diver-carrier (*L*) constructed of a small cardboard box and matchsticks (also shown in the cross section and in perspective). This carrier bears a wire arrangement to support two very small bowls, one for oil and one for lithium chloride* (*M* and *N*). These must be in such a position that they can easily be reached by the movable pipette."

* Lithium chloride solution was used as the flotation medium.

The gas is saturated with water vapor of the proper tension by passing through wash bottles of salt solution. The flow of gas through C and D is controlled by pinchcocks, and bubble counters are inserted. The gas stream through C is regulated to about one bubble per second, and through D to about 2–3 bubbles per second.

The manipulations are carried out as follows:

1. Place the bottom drop in the diver before placing the diver in the anaerobic filling apparatus.
2. Bring the diver into the apparatus and allow 5 min. for the gas in the diver to become equalized with that in the chamber (K).
3. During the latter part of the 5 minute period, bring the tip of C into the bulb of the diver and allow the gas stream to flush through the diver about 15 seconds.
4. Place the neck seals while the diver is in the apparatus, and the mouth seal after the diver has been removed from the apparatus. The diver is now ready for measurement.

In cases in which the exclusion of air is particularly important, as in experiments on anaerobic metabolism of tissue having a low oxygen consumption, place a drop of the diluted medium into the mouth of the diver under anaerobic conditions using an extra pipette for the purpose; this pipette need not be calibrated in any way. Then remove the diver from the apparatus and draw the mouth seal down into the neck by means of an "air pipette" (page 366) as follows:

(*a*) To prevent air from getting into the diver, pass the gas through the "air pipette" until the moment the pipette is to be used.

(*b*) The instant the "air pipette" is disconnected from the gas, connect it to the tube which the operator holds in his mouth and lower the tip of the pipette into the mouth seal. (A three-way stopcock connecting the air pipette with either the gas or the operator's mouth facilitates the operation.)

(*c*) Blow down the liquid which rises in the pipette by capillarity until the tip is completely, or almost completely, emptied. Then pierce the lower meniscus of the seal with the tip and extend it into the gas space under the seal.

(*d*) Suck out gas until the seal has been lowered to the desired position.

(*e*) Withdraw the pipette and rinse it at once to prevent clogging by evaporation and crystallization.

A test of the anaerobiosos may be made, as suggested by Boell, Needham, and Rogers (1939), by adding leucomethylene blue into a bottom drop under nitrogen and then sealing the diver. The drop will remain colorless for many hours if the filling has been properly conducted.

(10) MEASUREMENT

Saturating the Flotation Medium with Gas. The flotation medium should be saturated with air, unless, in a special case, it is considered necessary to saturate it with another gas. The air saturation should be carried out at least 1 hr. before introducing the diver in order to allow time for all of the bubbles to disappear. The aeration need not be performed before every experiment unless the experiments are conducted at pressures deviating considerably from the normal. The saturation is carried out by removing the flotation vessel from the thermostat, sealing the mouth of the vessel with the finger (not a stopper, since this would increase the air pressure when inserted), and shaking until the entire liquid is filled with air bubbles. The vessel is replaced in the thermostat, and at 5 min. intervals, the process is repeated twice.

If the medium is to be saturated with a gas other than air, the gas should be washed first in some of the medium or a salt solution of the same vapor tension and then passed through the medium to be used in a stream of fine bubbles.

When the ground-glass joint is fitted to the top of the vessel, the latter should be open to the outside through the manifold stopcock and the tap (*M*), Fig. 114 (page 349). This avoids suddenly compressing the air in the flotation vessel, which, if a diver is present, will cause undesirable displacements of neck seals.

The measurement is conducted by the following procedure:

1. Connect the air bottle with the outside for a moment through the threeway tap (*L*, Fig. 114), to equalize the pressure with that of the atmosphere.

2. Place the filled diver in the flotation vessel by either dropping it in, or, preferably, by lowering it in on the wire loop. Should air bubbles be attached to the diver, remove them with the wire loop or lift the diver out of the medium for a moment. If the mouth seal has been made correctly, an air bubble will not form at the mouth of the diver.

3. With stopcock *M* (Fig. 114) open to the atmosphere, place the ground-glass joint on the vessel and then turn the stopcock to connect the vessel to the manometer.

4. Adjust the manometer to make the diver float, and see whether the equilibrium pressure falls in the desired range. With practice, one can judge the equilibrium pressure by the rate at which the diver sinks. The position of the diver at the equilibrium pressure is arbitrarily taken as that at which the top of the upper air meniscus bordering the mouth seal coincides with the mark on the vessel or the cross hair in the observing microscope.

5. Again check to make sure no air bubbles are on the surface of the diver.

6. Rinse the mouth seal of the diver with medium as described on page 367 if small effects are to be measured and especially if narrow-neck divers with long seals are used. This need not be done if large changes in gas vol. are to be measured.

7. Use the manometer to place each flotation vessel under the pressure at which the divers are to be kept between measurements, *i.e.*, "the basic pressure." This pressure should be high enough to prevent the diver from rising and touching the flotation vessel. (Holter found that a pressure 3–5 cm. over the equilibrium pressure of the diver at the time is usually sufficient if the room temperature does not vary much.)

8. At the proper times, make measurements of the equilibrium pressure in the following manner:

(*a*) Adjust the manometer to the basic pressure before opening the stopcock of the flotation vessel.

(*b*) Slowly reduce the pressure until the diver begins to rise. Should the diver stick to the bottom so that it does not rise even at a pressure of about 10 cm. less than the equilibrium pressure, tap the neck of the flotation vessel lightly. Do not apply more negative pressure to make the diver rise, since this may cause too great a displacement of the neck seals. Tapping too hard may also disturb the seals.

(*c*) Regulate the pressure so that the diver remains at the equilibrium position for at least 10 sec., and then read the manometer to 0.5 mm.

(*d*) Repeat the adjustment and reading. If the diver does not change position during the time required for manometer readings

(about 30 sec.), displace it by means of the fine screw on the pressure regulator.

(*e*) If the two readings check, take the time of the second adjustment within 1 min. as the time of reading.

NOTE: Observe the shape of the menisci of the seals during measurement. If they do not wet the neck properly they are apt to be deformed and the accuracy of the measurement may suffer. It is sometimes helpful to vary the pressure quickly to about 10 cm. on each side of the equilibrium value to cause the menisci to move over the glass in their immediate locality and thus wet it. Of course it is necessary as a rule to employ control divers in each experiment. The use of the air bottle renders thermobarometer divers superfluous.

(11) REVIEW OF THE EXPERIMENTAL PROCEDURE

As an example of the steps in an actual experiment, Holter (1943, page 460) gave the procedure to be followed in a measurement of the respiration rate of echinoderm eggs. The experiment described was carried out in air, and two experimental divers and one control diver were used. The following is Holter's own description (page references, however, refer to this book):

Choosing the Proper Size and Type of Diver. Since the assumed oxygen consumption is large (about 5×10^2 µl./hr.), the diver need not be smaller than "standard size." The total gas volume of these divers being about 10 µl., the oxygen consumption will correspond to a change in equilibrium pressure of about 50 mm. per hour. As the oxygen used in the reaction drop is supplied only by diffusion, we need a reaction drop having a large surface and forming a thin liquid layer (this excludes filling *a* in the schedule on page 000. Naked echinoderm eggs do not stand direct contact with the surface of water (excludes filling *b*). No glass stopper is needed (excludes filling *e* and *f*) since the experiment is to be of short duration (2–3 hours) and since the pressure changes are expected to be so large that a slight gas loss by diffusion through the diver's mouth plays no appreciable role. On account of the magnitude of the anticipated effect we choose among fillings *c* and *d* the former, which also by its better utilization of the area of the diver bottom, gives the largest surface of the bottom drop.

Manipulations:

(1) If the flotation medium has not been in use for a long time the flotation vessel is, not later than 1 hour before the beginning of the experiment, shaken with air (page 374).

(2) The air bottle in the thermostat is connected to the outside air (page 374).

(3) The divers to be used are heated (page 353).

(4) Each diver receives 0.1 μl. of sea water as "forerunner" (page 362); if this does not satisfactorily moisten the inside wall of the diver an amount of sea water sufficient for such moistening is introduced into the diver bulb and sucked out again until 0.1 μl. is left (page 362). Until ready for use the divers are kept in an atmosphere saturated with sea water.

From this point on the description of the manipulations applies to only one diver.

(5) The centering of the pipettes (page 361) for sodium hydroxide, paraffin oil, and air (for placing the mouth seal) is checked and the two former pipettes are filled. Of these the sodium hydroxide pipette is filled last, and it is to be borne in mind that a small portion of the contents evaporates during the period before pipetting off. NaOH isotonic with sea water! (page 365).

(6) Immediately before picking up the organism in the braking pipette (page 359) the diver is mounted in the clamp stand (page 357).

(7) The organism is picked up, and the water which enters the pipette along with it is either adjusted to the mark or its length is measured (page 361).

(8) The braking pipette is mounted in its holder (page 361), the diver placed under it, the centering checked, and the pipette is emptied into the forerunner.

(9) The sodium hydroxide seal and the oil seal are placed in the diver neck (page 365).

If it is desired to check the contents of the diver under the microscope in order to determine whether the organism has suffered by the pipetting it is best done at this point, after the placing the oil seal.

(10) The mouth seal is placed (page 366).

(11) The diver is transferred to the thermostat, introduced into the flotation vessel (page 374), and freed from any air bubbles that may stick to its outside wall (page 374).

(12) The diver mouth is rinsed (page 375).

(13) The air bottle is shut against the outside air and manometer measurements are started (page 375). Each measurement lasts about 3 minutes.

(14) Upon completion of the manometric measurements the diver is removed from the thermostat and rinsed, whereupon the condition of the organism is checked under the microscope, best by submerging the entire diver in water.

(15) The neck seals are removed, the diver neck rinsed (page 364) and the organism removed from the diver (page 363).

(16) The diver is cleaned (page 353).

(17 The diver is dried (page 353).

It takes about 10–15 minutes to perform the manipulations 6–12. In a series of experiments, therefore, this is the time interval between the initial manometer measurements of the two subsequent divers.

(12) CALCULATIONS

The Diver Constant. A constant must be determined for each diver which represents the total gas volume (V) in the diver when the latter is at its equilibrium position at a given pressure (P) and temperature (t). V may be measured in two ways:

(1) By subtracting the total volume of the liquids in the diver from the total volume of the diver itself, and then correcting the gas volume obtained at barometric pressure and room temperature to P and t. The total volume of the diver is determined by weighing or filling from a burette.

(2) By calculating V according to the formula:

$$V = \frac{g_D + v_{oil}\phi_{oil} + v_w\phi_w - v_{oil}\phi_M - v_w\phi_M - (g_D\phi_M/\phi_{gl})}{\phi_M}$$

where g, v, and ϕ represent weight, volume, and density, respectively, and subscripts D, *oil*, w, M, and *gl* refer to diver, oil, aqueous phase, medium, and glass, respectively. This formula is derived from the expression which is based on the fact that, at equilibrium, the density of the diver unit equals that of the flotation medium:

$$\phi_M = \frac{g_D + v_{oil}\phi_{oil} + v_w\phi_w}{V + v_{oil} + v_w + g_D/\phi_{gl}}$$

In practice, the first method is less exact than the second for divers of the type described, because of the difficulty of measuring exactly the volume of mouth seals and the total volume of small divers by filling with a liquid. The chief reason for this is the poorly defined surface of the liquid at the mouth.

According to Lindestrøm-Lang (1943), the errors of the values in the formula for V which would result in a 1% error in V are:

v_{oil}	33%
v_w	50%
ϕ_{gl}	1%
ϕ_M	0.5%
ϕ_{oil}, ϕ_w	12%

For divers having a total volume of <5 μl., an accuracy of 0.02 mg. in (g_D) may be desirable, while for larger divers, an accuracy of 0.1 mg. is sufficient as a rule.

Holter (1943, page 464) has explained that the calculation may be simplified as follows: "Assuming that we are always using the same medium (which is generally true) and that it has the density ϕ_M, then the quantity $g_D - (g_D\ \phi_M/\phi_{gl})$ is a constant characteristic for each diver and may be calculated once for all and recorded in the diver inventory. Since, moreover, the same stock of paraffin oil will be used in all experiments it is likewise possible once for all to calculate the values of $V_{oil}\ \phi_{oil} -\ _{oil}\ \phi_M$ and to plot them as a function of V_{oil}. The same applies to the volume of the aqueous solutions, the densities of which in most cases deviate so little from 1 that they may be ignored. With these two curves drawn the whole calculation of V becomes a matter of reading the values of $V_w - V_w\phi_M$ and $V_{oil}\ \phi_{oil} - V_{oil}\ \phi_M$ from the curves, adding them to $g_D - (g_D\phi_M/\phi_{gl})$ and dividing by the value of ϕ_M which is also known once for all."

Change in Gas Volume. The pressure change (Δp) is read directly on the manometer. The equilibrium pressure (P) is actually the manometric pressure (p), plus the barometric pressure at the moment the air bottle was sealed, plus the hydrostatic pressure of the medium over the diver, plus capillary pressure at the boundary between the mouth seal and the gas in the diver neck. When calculating the change in equilibrium pressure (ΔP), this quantity may be made equal to (Δp) since all the other factors are essentially constant. The surface tension factor is eliminated by using sodium taurocholate in the medium. The relation $\Delta p = \Delta P$ requires the correction:

$$P = p\ (1 + 1000\ A/V_g')$$

where A is the cross-sectional area of the bore of the manometer tube, V_g' the vol. of the air bottle, and 1000 the barometric pressure in cm. of Brodie soln. For the dimensions of the manometer tube and air bottle given previously (pages 348–350) the correction amounts to about 1%.

When no correction is required for the solubility of the gas in the liquids in the diver, the following relationship holds:

$$\Delta V = V \Delta P/P_0$$

where P_0 is the normal pressure (1000 cm. Brodie soln.). However, the effect of the gas solubility is appreciable in many instances, and

Holter (1943, page 466) has chosen the following cases from Linderstrøm-Lang (1943) for which formulas for ΔV are given which are simplified and adequate for practical purposes:

Case 1. The solubility of all gases present is low. This case comprises all experiments in air, N_2 or O_2 wherein CO_2 does not occur or in which all the CO_2 formed disappears completely (respiration experiments in solutions without carbonate buffers, in which the CO_2 produced is absorbed by alkali). (Linderstrøm-Lang 1943, page 369).

In this case we find for all experimental procedures which may come into consideration in practice:

$$\Delta V = V \Delta P / P_0$$

Case 2. The gas to be measured is soluble in the liquids of the diver charge (CO_2). The amount of this gas is small ($\gtreqless 5\%$ of the total gas) in proportion to the other gases. The latter are sparingly soluble and do not change in quantity. This case includes *inter alia* all experiments which are based on the circumstance that acid is formed or disappears in a system containing carbonate buffer (Linderstrøm-Lang 1943, page 371).

In this case we find—under the assumption that the equilibrium pressure and the "basic pressure" differ no more than 50 cm. from each other, that the volume of the oil seal is small ($V_{oil} < 0.5\mu l$), and that narrow-necked divers with glass stoppers in the mouth seal are used:

$$\Delta V = \frac{V \Delta P}{P_0} \left(1 + \frac{v_W \alpha'_{CO_2}}{V}\right)$$

where v_w = the volume of the bottom drop, α'_{CO_2} = the absorption coefficient (not referred to 0^0, but to t^0) of CO_2 in the bottom drop at 760 mm. Hg and t^0 (for water at 22.5°, has the value 0.89; for definition of α'_{CO_2} (and β'_{CO_2}) see Linderstrøm-Lang (1943, page 366).

The insertion of ΔV and ΔP for the original differentials dV and dP instead of an integration is permissible also in this case, as shown by Linderstrøm-Lang (1943, page 371), (*i.e.*, the error involved is below 1%), provided the pressure change ΔP does not exceed 50 cm., Brodie solution—which it never does in practice.

Case 3. Like case 2, except that also one of the sparingly soluble gases (O_2) *varies in quantity.* This case includes respiration measurements in which the CO_2 evolved is not absorbed (Linderstrøm-Lang, 1943, pages 372 and 390).

In this case we find, under the same conditions as in case 2, augmented by the condition $v_w < 0.2\,V$, as well as under the assumption that the amount of CO_2 does not exceed 5% of the total gas:

$$\Delta V_{O_2} + \frac{\Delta V_{CO_2}}{1 + (v_W \alpha'_{CO_2} + v_{oil}\, \beta_{CO_2})/V} = \frac{V \Delta P}{P_0}$$

Where V_{O_2} and V_{CO_2} = the volumes of O_2 and CO_2 in the diver, β_{CO_2} the absorption coefficient (as above) of CO_2 in paraffin oil at 760 mm. Hg and $t°$

(at 24° = 0.91; also valid with sufficient approximation at 22.5°); the other designations as before.

The chief practical significance of this equation is that it permits of the determination of respiratory quotients (calculation of ΔV_{CO_2} when V_{O_2} is known from a parallel determination according to case 1). Since the determination of R.Q. calls for the highest possible accuracy, the correction term for the solubility of CO_2 in paraffin oil ($V_{oil}\ \beta'^{CO_2}$) is not disregarded in the above equation (as in case 2), though here too v_{oil} is small in relation to V.

The above formulas should be adequate for the calculation of most of the experimental combinations occurring in practice. If in special cases some of the experimental conditions to which the formulas correspond cannot be realized, or if it is desired to use other gas combinations than those mentioned, then the calculations of Linderstrøm-Lang (1943) will enable one to derive the corresponding equations.

Sample Calculation. In an experiment on the measurement of the respiration rate of the amoeba *Chaos chaos* Linné, Holter (1943, page 467) obtained the following data:

Diver No.	Weight, mg.	Total vol., µl.	ϕ_{gl}	Mouth diameter, mm.	Length of neck, mm.	Length of mouth seal. mm.
16 (amoeba)	18.07	18.1	2.40	0.69	10.0	2.6
16a (control)	14.70	14.7	2.40	0.71	10.3	3.0
17 (amoeba)	16.09	16.1	2.40	0.71	12.0	3.05

Bottom drop, 0.4 µl. (Pringsheim soln. plus amoeba).
Lower neck seal, 0.4 µl. (0.1 N sodium hydroxide).
Paraffin oil seal, 0.4 µl.

The curves in Figure 133 were plotted by Holter to show the time course of the oxygen consumption, and from the data he made the following calculations:

From the graphs:

Diver 16: Δp (1 hr.) = 2.43 cm.
Diver 17: Δp (1 hr.) = 1.83 cm.
Diver 16a (control diver) = constant.

Diver 16:

$$V = \frac{18.07 + 0.4\cdot 0.87 + 0.8\cdot 1.0 - 0.4\cdot 1.325 - 0.8\cdot 1.325 - \dfrac{18.07\cdot 1.325}{2.40}}{1.325} = 5.77\mu l.$$

$$O_2 \text{ consumption} = \Delta V = \frac{5.77\cdot 2.43}{1000}\ \frac{273}{273 + 22.5} = 12.9\cdot 10^{-3}\ \mu l./hr.$$

Diver 17:

$$V = \frac{16.09 + 0.4\cdot 0.87 + 0.8\cdot 1.0 - 0.4\cdot 1.325 - 0.8\cdot 1.325 - \dfrac{16.09\cdot 1.325}{2.40}}{1.325} = 5.11\ \mu l.$$

$$O_2 \text{ consumption} = \Delta V = \frac{5.11\cdot 1.83}{1000}\ \frac{273}{273 + 22.5} = 8.6\cdot 10^{-3}\ \mu l./hr.$$

The slight initial bend of the respiration curve for diver 17 has nothing to do with the respiration, it simply means that in case of this diver, which was the last one to be placed in the thermostat, the measurements were begun before the initial equalization of pressure and temperature had ended.

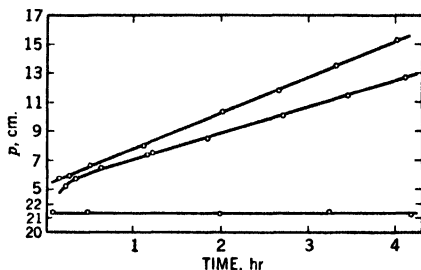



Fig. 133. Respiration of amoebae: p = pressure.
From Holter (1943)

(b) 0.1 Microliter or Capillary Diver Technique

Zeuthen (1943) developed a new type of Cartesian diver for respiration studies which has a gas volume about 100 times smaller than the μl. diver. These smaller divers, with a gas volume in the range 0.04–0.11 μl., were designated by Zeuthen as 0.1 μl. or capillary divers. Their smaller volume leads to a refinement of the measurement to the point where respiration intensities of 2×10^{-4} to 2×10^{-3} μl. oxygen per hour can be determined. In comparative studies with several divers Zeuthen observed an error of measurement of 2×10^{-5} μl. oxygen per hour, while variations in the respiration of the individual cell during an experiment were much less. Although the greatest respiration rate thus far measured in capillary divers is 2×10^{-3} μl. oxygen per hour, Zeuthen is of the opinion that all rates less than 10^{-2} μl. oxygen per hour should be measured in capillary divers of suitable dimensions, while higher rates should be followed in μl. divers. Aside from the design of the diver pipettes, and the flotation vessel, the apparatus is the same as that used with the μl. diver.

(1) THE CAPILLARY DIVER

Dimensions. The diver (Fig. 134) which is chosen as an example has a gas vol. of 0.072 μl. It is closed with seals of flotation medium (M_1 and M_2). The medium is that of Holter having a

specific gravity of 1.325 (page 347); however, it contains 0.5% sodium taurocholate and has been made 0.1 N with respect to sodium hydroxide by adding the calculated amount of 7.35 N sodium hydroxide (29.4 g. sodium hydroxide in 100 g. soln.), which also has a specific gravity of 1.325.

The length of the various columns in the diver is shown in Figure 134. In general, the lengths of the various components in the charged diver should fall in the range (given in mm.):

Seals of medium (M_1 and M_2) .... 0.7 to 1.0
Air Space (L_1) .... *ca.* 0.5
Solid paraffin seal (P) + paraffin oil seal (PO_1) .... 0.7 to 1.0
Water drop (W) containing respiring cell .... 0.2 to 0.3
Air space (L_2) 1.5 to 2 times the diameter of the diver capillary
Oil seal (PO_2), between menisci .... 0.02 to 0.03
Air space (L_3) .... 2 to 3

The paraffin oil seals prevent loss of water from W. The solid paraffin fixes the positions of the various columns. The oil seal (PO_2) is made short so that the carbon dioxide formed by the respiration can diffuse from W to M_2 becoming absorbed due to the alkalinity of the latter.

Preparation of the Diver. The divers were first made in the Carlsberg Laboratory from Thuringer glass, different samples of which proved to be rather variable in property. Later Jena glass was used and it was found that the glass drawn in an ordinary flame (600–700°) was too brittle but that glass drawn at 1200–1400° was suitable. The final test of the suitability of a glass is the preparation and testing of control divers made from it. No information is available at the moment concerning the properties of American-made glass for divers.

The tubing from which the diver capillaries are drawn should be thin-walled (outer diameter/inner diameter = about 1.25). Only capillaries should be selected which have constant outer diameters, as tested by a gauge such as the Zeiss cover slip gauge. For those selected, the ratio of the weight of the glass to the weight of mercury required to completely fill each tube is determined. With Jena glass (sp.gr. *ca.* 2.40) Zeuthen reported that the following requirement must be met for the weight of the mercury and glass: 9.2 > mercury/glass > 7.7, and with Thuringer glass: 10.0 > mercury/glass > 8.3. Variations of the inner diameter must be checked by moving a 5–10 mm. column of mercury along the capillary and

determining its length at different positions. Capillaries varying in bore less than 2–3% and having lengths of more than 5 cm. should be chosen. The inner diameter is finally measured by weighing the mercury needed to fill the capillary completely. Divers are cut 5–6 mm. long, their length is measured, and then they are stored in numbered test tubes.

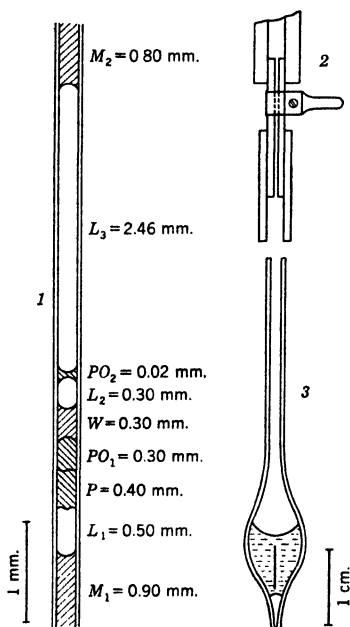



Fig. 134.
1. The "standard diver":
length = 6 mm.,
diameter = 0.17 mm.,
gas space = 0.072 μl.
The data in the figure apply to lengths of the individual spaces and seals.
2. Connecting piece between flotation vessel and manometer.
3. The flotation vessel.

From Zeuthen (1943)

Cleaning the Diver. Zeuthen has given the following procedure for cleaning the diver:

1. Hold the diver in cork-tipped forceps under water and use a thin glass rod to push out seals and air bubbles.
2. Place the diver in a cylinder of glass-distilled water and suck the diver into a pipette having an inside diameter of about 1 mm. for a distance of 2–3 cm. from the tip, and a constriction at the 2–3 cm. point so that the diver cannot pass. Continue to draw water up into the pipette in order to flush the diver which is held at the construction.
3. Place the tip of the pipette at a slant against the bottom of

the water cylinder and blow out the water from the pipette while retaining the diver in the pipette.

4. In a similar fashion, flush the diver with alcohol, toluol, alcohol, and glass-distilled water in the order given.

5. Transfer the diver into the tube in which it is to be stored, draw off excess water, and dry in an oven at 110–120°.

6. Never touch the cleaned diver with the fingers.

(2) THE FLOTATION VESSEL

The flotation vessel (Fig. 134) has been so designed that the diver floats in medium enclosed between two air spaces, and the distance between the upper and lower surfaces of the medium is such that the ends of the diver are only about 0.5 mm. from the nearest air space. With this design it has been found that the seals (M_1 and M_2) are effective in minimizing the exchange of air between the gas phase of the diver and the medium, for the reasons discussed by Zeuthen (1943, page 483).

(3) PIPETTES

Braking Pipette. The form of braking pipette required for filling the capillary diver is shown in *A*, Figure 135. The capillary (I) has an outer diameter of about 0.5 mm., and an inner diameter a good deal less than 0.1–0.2 mm., and a length of about 3 cm. One end of it is drawn out in a micro flame to an exceedingly fine bore, which allows water to rise in the capillary at a rate of about 0.5 cm. per sec. when the wide end is dipped into water to test it. The tube (II), which is 1.5 mm. wide, holds I by means of a bit of DeKhotinsky cement. A thin-walled rubber tube is connected to II. A block of soft transparent crude rubber (III) (a piece of red rubber laboratory tubing may be used) about 2 $\times$ 3 $\times$ 5 mm. is cut from a larger piece after the rubber has been pierced with a needle and attached to the wide end of I as shown. At the end away from I, the block is cut at a slant to aid in finding the hole under the microscope. The capillary diver is fitted into this hole using a binocular microscope with good lighting to lessen the danger of breaking the diver during the operation. The procedure to be used is as follows:

1. Pick up the diver by means of two watchmaker's forceps which have their points covered with cork.

2. Push the diver into the rubber block (III) until it almost touches the glass of I.

3. Test for tight fitting by dipping the open end of the diver into alcohol, blowing through the rubber tubing attached to II, and then sucking a small drop of alcohol into the diver. If the drop is not movable with equal ease in both directions, change the position of the diver in III and repeat the test with alcohol until tight fitting is obtained.

4. Finally, blow out the alcohol and allow the diver to dry for a few min.

The Ball-Tipped Pipette. This pipette is very much the same as the one used with microliter divers (page 360), except that it is drawn out to an extremely fine thin-walled capillary. The ball is formed by passing the pipette tip through a micro flame while compressed air at 0.3–2 atmospheres is connected to the pipette. The greater the pressure, the larger the hole that will be blown in the ball, and the longer the flame, the larger the ball itself will be. The dimensions, relative to the diver capillary are apparent from *D*, Figure 135.

(4) FILLING THE CAPILLARY DIVER

Determining the Length of the Seals of Medium (M_1, M_2) to be Used. First, the length of the column of medium (l_M), which placed in the diver will make the otherwise empty diver float, should be calculated. It is most convenient to work with divers in which:

$$l_M = (0.4 \text{ to } 0.5)\, l_D$$

where l_D is the length of the diver. The value of l_M may be calculated from the following formula which was derived from that given by Linderstrøm-Lang (1943, page 363):

$$l_M = l_D \left(1 + \frac{13.56}{\phi_{gl} f} - \frac{13.56}{\phi_M f}\right)$$

where f is the ratio of the weight of mercury to glass which was determined in the selection of the diver capillary (page 383) and ϕ_{gl} and ϕ_M are the densities of the glass and medium, respectively (page 354) ($\phi_M = 1.325$).

As will be described later, the columns W, PO_1, P, and PO_2 are first placed in the diver in the order given. M_1 and M_2 are placed last. The following conversion factors are used for the calculation of the combined length of M_1 and M_2:

1 mm. water replaces 0.75 mm. medium
1 mm. paraffin or paraffin oil replaces 0.65 mm. medium

Zeuthen (1943, page 501) employed the following example for a diver having a diameter of 0.17 mm. and a charge as shown in *A*, Figure 134:

$l_D = 5.87$ mm.
$l_M = 2.15$ mm. (calculated)
$P + PO_1 = 0.54$ mm.
$W = 0.26$ mm.
$L_2 = 0.37$ mm.
$PO_2 = 0.05$ mm.
$P + PO_1 + PO_2 = 0.59$ mm. (which replaces 0.38 mm. medium)
$W = 0.26$ mm. (which replaces 0.20 mm. medium)

Total: 0.58 mm. medium

$M_1 + M_2 = 2.15 - 0.58 = 1.57$ mm.
M_1 chosen 0.82 mm.
M_2 chosen 0.75 mm.

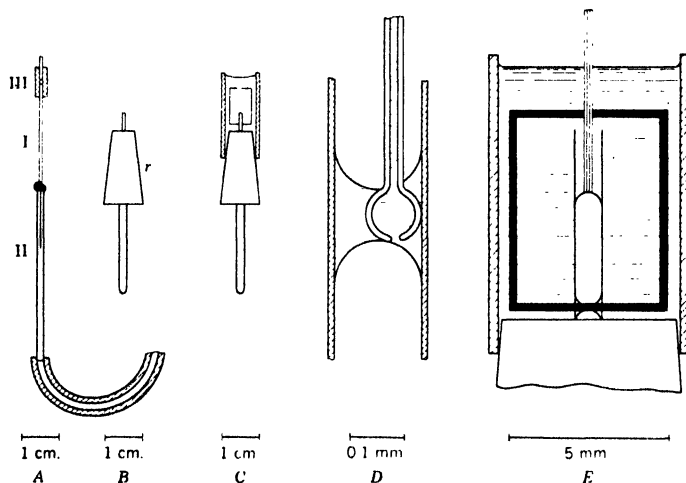



Fig. 135. The technique of filling the diver.
From Zeuthen (1943)

"The equilibrium pressure of the diver is here 1 atmosphere —12.5 cm. H_2O. Most frequently, however, it will be found that the equilibrium pressure at the first filling of the diver is rather far removed from 1 atmosphere. When the diver floats it is possible to measure M_1 and M_2 accurately at the equilibrium pressure. The figures found in this way are used in calculating M_1 and M_2 at the next filling. As a rule we find that the diver can be filled in such a way that the equilibrium pressure at the beginning of the experiment is 1 atmosphere ±40 cm. H_2O (in recent experiments, though, 1 atmosphere ±20 cm. H_2O)."

Placing W, PO_1, and P. With the diver attached to the rubber block of the pipette, the biological sample in its aqueous medium W is drawn in, followed at once by the short seal of oil PO_1. The filling is carried out under the microscope. The liquids should be in small narrow cylinders which prevent the rubber block from coming into contact with them. Speed is essential in bringing in the oil seal after W has been taken in, since Zeuthen has found that if W is exposed to evaporation for not more than 10 sec. at about 50% humidity a loss of less than 10% of W will be incurred. It is advisable to work in a moist chamber in order to reduce evaporation losses.

The paraffin seal (P) is next placed by pushing the end of the diver through a layer of paraffin 0.2–0.3 mm. thick. The paraffin (m.p. 58–60°) must be absolutely clear and white; the layer is formed by kneading the material between the fingers. It is then placed on a piece of rubber to minimize damage to the diver when piercing the paraffin. The diver is held with the forceps and wiped off to remove any paraffin or oil from the outside. A small blunt glass rod which can go into the diver is used to push in the P, PO_1, W combination about 1.2–1.5 mm. to leave space for M_1. This must be done under the microscope and very slowly. Caution is required to prevent the sample in W from coming into too intimate contact with the air-water interface or particularly the oil-water interface. Now the brake is clamped vertically and the length of the diver is measured, if this has not been done previously. The transparency of the rubber block permits the end of the diver to be seen.

Placing PO_2. A rubber stopper with dimensions approximately the same as those in B, Figure 135, is fastened on its wide end to a glass rod by which it may be clamped. A slit, 2–3 mm. deep, is cut into the surface of the small end of the stopper and the end of the diver is placed in this slit. With the diver mounted vertically in this fashion, the ball-tipped pipette is clamped directly over it, and the clamp holding the rubber stopper is raised so that the tip of the pipette enters the diver. The ball is brought to the point where PO_2 is to be placed and the oil is carefully blown out of the pipette to form the seal. A slight jerk is used to disengage the ball from the seal and then the pipette is withdrawn from the diver entirely. The flexibility of the pipette stem makes strict alignment between the

pipette and diver less important than it is when microliter divers are used.

The ball-tipped pipette may also be used to place oil in direct contact with *W*, if desired. This is accomplished by moving the ball very close to *W* so that the oil will be able to come into contact with *W* before a separate seal can be formed. The thickness of such an oil layer can be regulated by drawing back into the pipette any excess oil.

Placing M_2. A glass tube is fitted on the rubber stopper (*r*) to form a vessel around the diver as in *C*, Figure 135. The vessel is filled with flotation medium to 2–3 mm. over the top of the diver. In order to facilitate observation under the microscope, a square piece of cover slip is cemented on the tube with Canada balsam and the edges of the cover slip are held with DeKhotinsky cement to prevent it from sliding off (*E*, Fig. 135).

M_2 is then placed by withdrawing air from the end of the diver through the tip of a pipette, arranged as shown in *E*, Figure 135. As the tip is brought into the end of the diver, gentle suction is applied so that the end of the tip is kept at the surface of the medium. In this way, the length of M_2 will be equal to the distance the end of the pipette tip is brought into the diver.

Zeuthen (1943, page 506) has described the process of placing a 1.00 mm. M_2 as follows:

"Before the pipette is introduced into the diver, a horizontal line in the ocular of the microscope is brought to intersect with the image of the pipette 1.00 mm. from its tip; in placing M_2, the diver, encircling the pipette, is raised so much that its edge is just level with the line chosen in the ocular. After making sure that actually a current of medium has passed from the surrounding liquid through the diver's neck into the pipette, the latter may be withdrawn from the diver, emptied by blowing, and rinsed with water."

Placing M_1. The medium in the vessel surrounding the diver is poured out; the diver is inverted and the M_2 end is placed in the slit. The vessel is again filled with medium, and the ensuing procedure is the same as that used for placing M_2.

Transfer of Diver to Flotation Vessel.

1. Remove diver from the slit and allow to float in the medium over the rubber stopper with the M_2 end upward.
2. Draw the diver up into an ordinary pipette having a drawn-out tip, along with about 0.5 ml. medium. Keep the M_2 end up.

3. Make the diver go up and down quickly a few times by sucking and blowing to free it of air bubbles which might be adhering.
4. Pipette the diver and enough medium into the flotation vessel so that the column of medium exceeds the length of the diver by about 1 mm.

Replacement of M_2. Some evaporation of M_2 during the filling of the diver necessitates replacement of the medium in M_2 in order that its density be the same as that of the surrounding liquid. The replacement is effected by applying alternate suction and pressure three times to the flotation vessel. The air space (L_3, Fig. 134), which is next to M_2, is relatively large and the expansions and contractions of the air force the medium out and then into the end of the diver. The effect on M_1 is naturally very much less. The process of filling the diver and placing it in the flotation vessel takes 20–30 min.

(5) MEASUREMENT

For the observation of the diver during an experiment, Zeuthen used the horizontal microscope with the vertical micrometer movement illustrated in Figure 151. A particular mark on the diver, such as one of the menisci, is made to coincide with a reference mark in the ocular of the microscope to establish the equilibrium position. For the finest measurements, the diver is kept in a floating position throughout the experiment. This is not very difficult, since the motion of the diver is quite slow when pressure changes on the medium fall within 1–2 mm. (water) of the equilibrium pressure. In experiments in which the greatest accuracy is not required, the diver may be left to sink to the bottom of the medium after a measurement. Should the diver stick to the lower medium-air interface, making it difficult to raise it, gentle tapping of the flotation vessel will free it. Before taking another reading, the diver should be made to float for a fixed interval (about three minutes) to allow time for complete equilibration of the pressure.

The equilibrium pressure is taken as the mean of the pressures required to cause the diver just to begin to rise and to sink. The two pressures are within 1–3 mm. water from one another, and the readings require about one minute. While the accuracy of microliter diver measurements is ± 0.5 mm. water, that of the capillary diver is ± 1 mm.

After the experiment the diver is immediately removed from the flotation vessel by filling the latter with water and pouring out the water with the diver into a small cylinder. Using a microscope, the lengths of L_1, L_2, and L_3 are measured, taking the greatest distance from meniscus to meniscus. The boundary between L_1 and P is not a meniscus; hence there will be five menisci in a diver such as that in *A*, Figure 134.

(6) CALCULATION

Considering the menisci to be hemispherical, the gas volume V of the diver at the barometric pressure prevailing at the end of the experiment is:

$$V = (l_1 + l_2 + l_3)\pi r^2 - \frac{5\pi r^3}{3}$$

where l represents the length of L, and r is the radius of the diver capillary.

At the floating position the volume is $V \times B/B - p_2)$, where B is the barometric pressure (mm. water) and $(B - p_2)$ the final equilibrium pressure observed. No correction for the height of the rise of the medium in the diver capillary is necessary.

When ΔP is the change in the equilibrium pressure (mm. water), the oxygen consumption (ΔV) in a respiration experiment is:

$$\Delta V = \frac{\Delta P}{10{,}300} \cdot V \frac{B}{B - p_2}$$

or corrected to standard conditions:

$$\Delta V = \frac{\Delta p}{10{,}300} \cdot V \cdot \frac{B}{B - p_2} \cdot \frac{B - p_1}{10{,}300} \cdot \frac{273}{273 + t}$$

where $(B - p_1)$ represents the average pressure of measurement during the given period.

Usually the corrections are small enough to render it sufficient to calculate the change in vol. by the formula:

$$\Delta V = V \frac{\Delta p}{10{,}300}$$

where ΔV and V are given in microliters and p in millimeters water per hour.

(7) CHOICE OF DIVERS FOR DIFFERENT RESPIRATION RATES

The choice of divers for measurements of respirations of approximately known intensities should follow the general considerations given by Zeuthen (1943, page 509):

1. The changes in equilibrium pressure should fall in the range of 3–10 cm. water per hour (2–20 if necessary).
2. The respiration rate must not be great enough to move (PO_2) toward W. This can be checked by observation through the microscope.
3. Total changes in equilibrium pressure over 50 cm. water (corresponding to a drop in oxygen in the diver from 21 to 16%) should be avoided.
4. It is well to calculate, for the diver dimensions used, the magnitude of the respiration rate which would result in the danger of oxygen deficiency (perhaps even the carbon dioxide absorption should be calculated according to the formulae of Zeuthen, 1943, pages 492–494). The following formula is used to relate the dimensions to the respiration rate:

$$l = \frac{0.11 A \sigma_{O_2}}{R} - L_D \frac{\sigma_{O_2}}{\sigma_{1O_2}}$$

where l (in mm.) is the greatest length of W permissible for the respiration intensity R (in μl./hr.), A is the cross-sectional area of the diver capillary, in mm.^{2} σ_{O_2} is the "standard rate of passage" for oxygen through water at 20°; its value is 0.204. σ_O is tqe "standard rate of passage" for oxygen through paraffin oil to which the value, 0.10, has been ascribed; and L_D, in mm., is the average length of the diffusion path through PO_2, which is defined as $L_D = L + (2r/3)$, where L is the shortest distance between the oil menisci and r is the radius of the diver capillary. Thus:

$$l = \frac{A}{R} \times 0.11 \times 0.204 - \left(L + \frac{2r}{3}\right) \frac{0.204}{0.10}$$

When there is a tendency to condensation of moisture on the walls of L_2, when W consists of fresh water, a film of oil about 0.05 mm. thick is placed on W. Then:

$$l = \frac{A}{R} \times 0.11 \times 0.204 - \left(L + \frac{2r}{3} + L_0\right) \frac{0.204}{0.10}$$

where (L_o) is the thickness of the oil film.

For a W of 0.2–0.3 mm. in the diver (*A*, Fig. 134), 10^{-8} μl./hr. is the maximum respiration intensity which can be determined.

5. When the diameter of the diver is about 0.02–0.03 mm. greater than the diameter of the cell, the change in equilibrium pressure will usually be large enough even for weakly respiring cells. The smallest glass divers which have been used have a diameter of 0.13 mm., and the smallest cells whose respiration can be measured (about 10^{-4} μl./hr.) will therefore be around 100 to 50 μ in diameter for weak and strong respirations, respectively.

(c) *Methods Other Than for Respiration*

CHOLINESTERASE

Linderstrøm-Lang and Glick (1938) developed a Cartesian-diver method for the measurement of cholinesterase based on the principle of the method utilizing the Warburg apparatus (Ammon, 1933). This principle depends on the fact that if the enzymatic scission of a choline ester proceeds in a bicarbonate buffer, the acid liberated will cause an equivalent evolution of carbon dioxide which can be measured gasometrically. Some investigators use a bicarbonate-Ringer medium while others employ only a bicarbonate solution for the estimation of the enzyme. The advantage of the presence of the other salts which are in the Ringer solution is that they activate the enzyme. By substitution of other ester substrates, the present method can be applied to the measurement of lipolytic enzymes, atropinesterase, etc. For a titrimetric method see page 310.

Linderstrøm-Lang and Glick Method for Cholinesterase

SPECIAL REAGENTS

Buffer Substrate Solution. Prepare 0.5% acetylcholine chloride in bicarbonate-Ringer soln.

Bicarbonate Ringer Solution (*p*H 7.4). Add 2 ml. of 1.2% potassium chloride, 2 ml. 1.76% calcium chloride ($CaCl_2.6H_2O$), and 20 ml., 1.26% sodium bicarbonate to 100 ml. 0.9% sodium

chloride. Saturate the soln. with a mixture of 5% carbon dioxide and 95% nitrogen.

PROCEDURE

1. Pipette 1 μl. buffer-substrate soln. into the bottom of a microliter diver.
2. By means of a pipette extending nearly to the surface of the soln. in the diver, pass a rapid stream of 5% carbon dioxide in nitrogen through the diver for 30 sec.
3. Pipette 0.3 μl. enzyme soln. into the bottom drop in the diver and again pass the carbon dioxide-nitrogen mixture through the diver.
4. Place the paraffin oil seal in the neck of the diver followed by the mouth seal.
5. In a parallel fashion, prepare a control diver containing 0.3 μl. inactivated enzyme or soln. without enzyme.
6. Proceed with the diver technique as described on pages 342–382.

NOTE: According to the work of Linderstrøm-Lang and Holter (1942), the relatively high permeability of paraffin oil and flotation medium to carbon dioxide would make it advisable to use oil and medium which are saturated with the carbon dioxide-nitrogen gas mixture. The apparatus illustrated in Figure 132 would be useful for charging the divers.

THIAMINE AND COCARBOXYLASE

The method of Ochoa and Peters (1938) for the estimation of thiamine and cocarboxylase depends on the stimulating effect the compounds have on the decarboxylation of pyruvic acid by alkaline-washed yeast. Employing the Warburg technique these authors were able to determine down to 0.01 μg. cocarboxylase and 0.05 μg. thiamine. By adapting this method to the Cartesian μl. diver, Westenbrink (1940) increased the sensitivity to the measurement of 0.05 mμg. cocarboxylase and 0.5 mμg. thiamine. The sensitivity of the method varies with the variety of the yeast and the manner in which it is washed. The thiamine determination may be carried out most suitably when the concentration of cocarboxylase present is small compared to that of the thiamine. For instance, in one experiment, thiamine had no influence on the carbon dioxide evolution

when more than 0.01 μg. cocarboxylase was present; however, 0.001 μg. proved to be a favorable amount.

There is little reason why the Cartesian diver could not be applied to the method of Atkin *et al.* (1939) and Schultz *et al.* (1942), which depends on the stimulation of yeast fermentation by thiamine.

Westenbrink Method for Thiamine and Cocarboxylase

SPECIAL REAGENTS

Yeast Suspension. Stir 100 mg. yeast with 5 ml. 0.1 *M* secondary sodium phosphate for 4 min. at 16–20°. Centrifuge 1 min.; discard supernatant and repeat the procedure twice. Finally wash residue once with 5 ml. water in the same manner. To the residue now add 0.12 ml. of 0.1 *M* magnesium chloride and enough 0.1 *M* phosphate buffer, *p*H 6.2, to bring the total vol. to 1.2 ml. Use soon, since the treated yeast deteriorates rapidly even in the cold.

1.0 M Sodium Pyruvate in 0.1 *M* phosphate buffer, *p*H 6.2.

Thiamine (0.8 mg. per ml.) in 0.1 *M* phosphate buffer, *p*H 6.2.

Cocarboxylase (0.005 mg. per ml.) in 0.1 *M* phosphate buffer, *p*H 6.2.

PROCEDURE

1. Pipette into a Cartesian diver, having a vol. of 20–30 μl., 0.2 μl. portions of the pyruvate, thiamine and cocarboxylase solns. When thiamine is to be determined, use the cocarboxylase soln. with 0.2 μl. unknown; and when cocarboxylase is to be measured use the thiamine soln. with the unknown.
2. Set up a control experiment by replacing the unknown soln. by the phosphate buffer.
3. Place divers containing the reagents in glass tubes closed by rubber stoppers to prevent evaporation.
4. Prepare the yeast suspension and add 0.5 μl. of it to the solns. in the divers.
5. Seal the neck of each diver with 1.7 μl. mineral oil; place diver in the apparatus and take readings of the gas evolution after 30 min.
6. Calibrate the measurements by comparisons with those obtained using known amounts of the constituent being analyzed.

7. For the determination of both components in a mixture, first measure the cocarboxylase. Then determine the thiamine in a separate experiment by adding, to the 0.2 μl. unknown, 0.2 μl. cocarboxylase soln. containing sufficient of the substance to make the total in the diver 0.001 μg.

DIPHOSPHOPYRIDINE NUCLEOTIDE

Anfinsen (1944) adapted the method of Jandorf, Klemperer, and Hastings (1941) to the measurement of diphosphopyridine nucleotide (DPN) in microtome sections of tissue by the use of the Cartesian-diver technique. The method of Anfinsen permits the estimation of 1–6 mμg. DPN with an error of less than 5%; this represents a thousandfold increase in the sensitivity of the Warburg procedure of Jandorf *et al.* The principle of the method lies in the fact that the enzymatic conversion of hexose diphosphate to phosphoglyceric acid and phosphoglycerol can be made to take place under conditions in which the quantity of DPN present is the limiting factor. These conditions require the presence of a muscle extract to supply enzymes and arsenate to limit specifically the glycolytic process. The dearsenylation of the 1-arseno-3-phosphoglyceric acid formed occurs spantaneously. The molecule of acid produced in this step is made manifest by the liberation of carbon dioxide from a bicarbonate buffer; thus the gas evolution becomes a measure of the concentration of the DPN.

Anfinsen Method for Diphosphopyridine Nucleotide

Extraction of DPN from Tissue Sections. Since DPN is rapidly destroyed by concomitant enzymes in the tissue, the following procedure is employed for the extraction:

1. Weigh frozen-dried sections on a quartz torsion balance (page 191) and transfer to micro centrifuge tubes (Fig. 136 *A*), made of 3 or 4 mm. tubing.
2. Draw out tube to form a constriction (*B*).
3. Add a known amount of distilled water with a constriction pipette (page 172) and rapidly seal the tube (*C*).

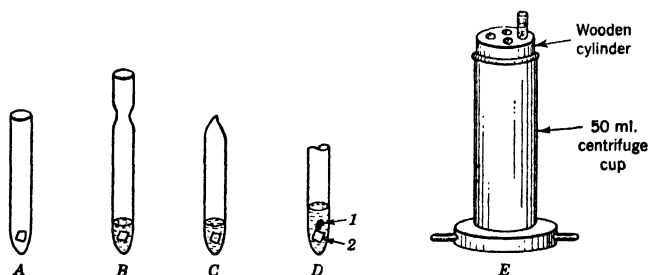



Fig. 136. Apparatus used in extraction of DPN from frozen dried sections: *A*, micro centrifuge tube with frozen dried section; *B*, tube after addition of water; *C*, tube sealed off during heat inactivation; *D*, tube during extraction of DPN. *From Anfinsen (1944)*

4. Plunge sealed tube into boiling water, and, after 2 min., remove and chill well in ice water.

5. Centrifuge down drops of soln. left on the sides and top. The special centrifuge cup (*E*) is convenient for this purpose. Open tube with a file.

SPECIAL REAGENTS

0.154 M Sodium Bicarbonate saturated with a mixture of 5% carbon dioxide and 95% nitrogen.

0.003 M Disodium Hydrogen Arsenate.

0.016 M (approx.) Sodium Hexose Diphosphate (≈ 1 mg. phosphorus/ml.). Prepare from the calcium salt by adding about 700 mg. of the latter to 40 ml. of 1% oxalic acid. After shaking, neutralize the mixture with sodium bicarbonate to chlorophenol red. Decolorize with charcoal and filter. Test filtrate for oxalate, and, if present, precipitate it with a little solid calcium hexose diphosphate and refilter through the original filter paper. Determine the phosphorus content of the filtrate and dilute the soln. until it contains 1 mg. organic phosphorus/ml. The presence of 3–4% inorganic phosphorus may be ignored. Store in refrigerator where the soln. will be stable for several months.

Muscle Extract. Remove the fascia as well as possible from muscles of the hind legs and back of a cat. Homogenize thoroughly with an equal weight of water and crushed ice, and centrifuge. A Waring blendor and a Sharples centrifuge are useful for these steps. With stirring, add 4 vol. ice-cold acetone to the soln. in a slow stream. Let the precipitate stand for 30 min. in the cold; decant the supernatant, and centrifuge the remainder. Wash the residue twice with cold acetone in the centrifuge, transfer to a Büchner funnel and wash with acetone and ether. Break up the cake into small pieces and dry overnight *in vacuo* over sulfuric acid. Prepare a paste of this acetone powder by grinding 600 mg. with successive 2 ml. portions of water until 20 ml. has been added. Centrifuge; dialyze the supernatant liquid in the cold against running distilled water for 24 hr., and centrifuge. Remove interfering nucleotides by adding 600 mg. of Norit charcoal to the dialyzed soln. Shake mechanically in the cold for 1 hr., centrifuge, and treat the supernatant fluid with another portion of charcoal for 1.5 hr. Centrifuge and filter. The DPN blank for this light brown soln. is small at first and disappears completely after 12–24 hr. at 6°. The acetone powder is very stable when kept *in vacuo* in the cold. The activity of the aqueous extract remains constant 4–5 days when kept cold.

PROCEDURE

1. Combine 0.4 ml. bicarbonate soln. with 0.6 ml. hexose diphosphate and 0.3 ml. arsenate. To 1 vol. of this mixture add 0.17 vol. DPN soln., either the standard or unknown soln.
2. Pipette 1.60 μl. resulting soln. into the diver.
3. Make up to a total vol. of 3.14 μl. in the diver with water and muscle extract (1.0 ml. muscle extract equivalent to 30 mg. acetone powder). About 1 vol. muscle extract, diluted with 0.5 vol. distilled water will probably be required.
4. Flush the diver with the gas mixture containing 5% carbon dioxide and 95% nitrogen for 3 min. to bring the *p*H to 7.4.
5. Seal the neck of the diver with paraffin oil saturated with the gas mixture and transfer to the flotation tube.

6. Take readings at 5 min. intervals after allowing 5 min. for the initial equilibration.

2. Optical-Lever Respirometry

Heatley, Berenblum, and Chain (1939) developed an apparatus in which a respiration chamber of 40–80 μl. is ground into a glass plate and covered with a mica membrane to which mirrors are attached. A change of gas pressure within the chamber causes the

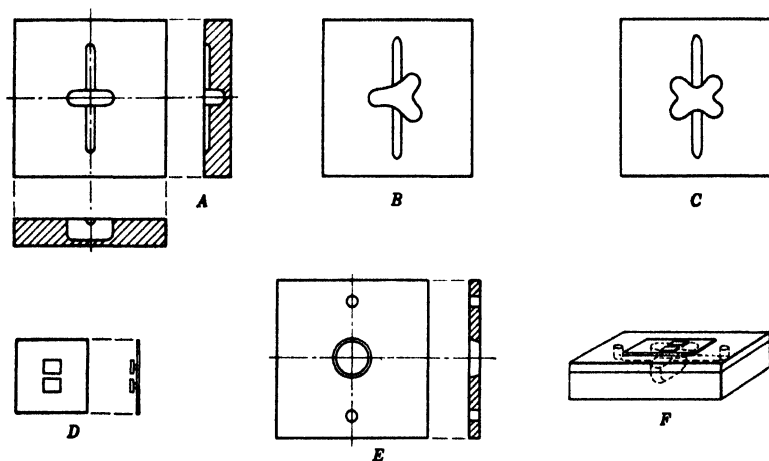



Fig. 137. Component parts of respiration chamber: *A, B, C,* cups with cavities for two, three, and four separate droplets, respectively; *D,* mica membrane with mirrors attached; *E,* "plate"; *F,* complete assembly ready for placing in brass case. *From Heatley (1940)*

mica to bulge and a compensating external pressure can be applied to restore the membrane to its original position as indicated by an optical lever. From the volume of the gas space and the change in pressure required to keep it constant, the change in the gas volume may be calculated. The instrument is sufficiently sensitive to measure changes in the gas volume of 1 μl. per hour (about 200 times as sensitive as the usual Barcroft or Warburg apparatus).

Heatley (1940) described an improved model in which up to six respiration chambers may be mounted on a revolving frame to

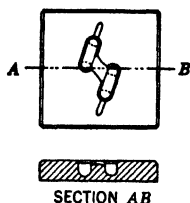



Fig. 138. Respiratory chamber.
From Berenblum, Chain, and Heatley (1940)

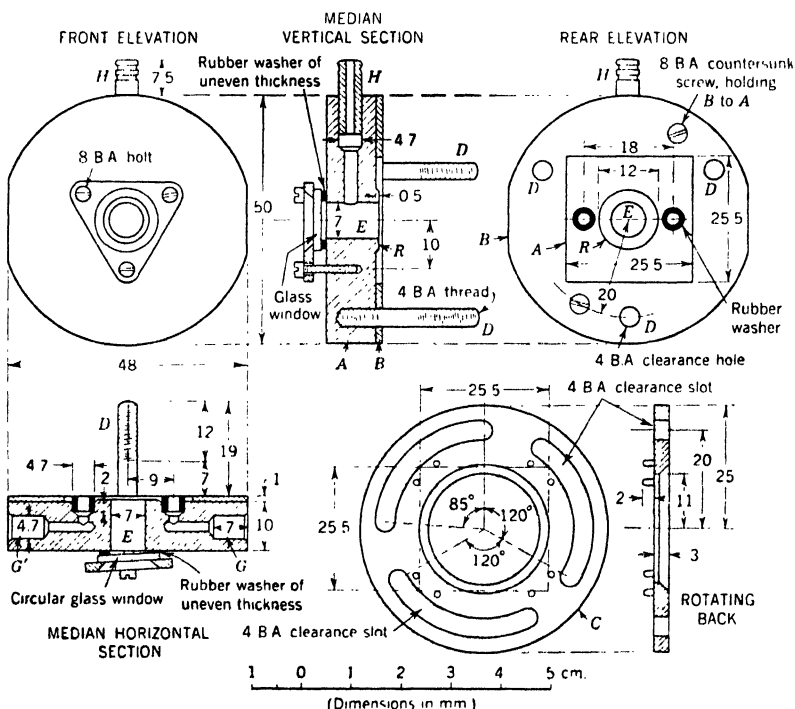



Fig. 139. Details of metal case for respiration chamber.
From Heatley (1940)

facilitate manipulation and measurement. Before the war the apparatus was made by *Unicam Instruments Ltd.*; but at the date of

this writing production of the instrument has not yet been resumed. The apparatus is rather complicated, mechanically, and since there are other more available instruments which have simpler construction and greater sensitivity the use of the optical-lever manometer will probably be limited. Therefore, only a cursory description of the apparatus will be given.

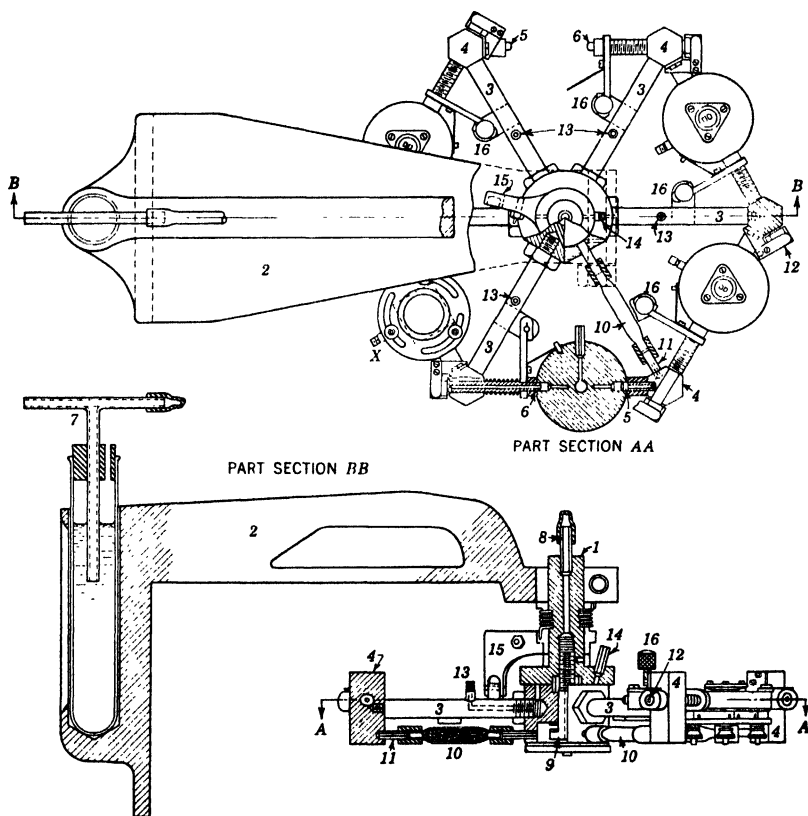



Fig. 140. Details of revolving frame.
From Heatley (1940)

The respiration chamber (Fig. 137) consists of a "cup" which is a 25 mm. square of glass, 3–4 mm. thick, in which cavities are ground and then coated with a paraffin film. Drops placed in these

cavities will not flow together, but may be mixed by means of a magnetic "flea." The "cups" are covered with glass "plates" containing three holes, as shown in Figure 137. The large hole in the "plate" is covered with a 12 mm. square mica membrane not over 18 μ thick. Two 1 mm. squares of cover slip glass are cemented to the mica with Seccotine, and mirrors, 2 × 3 mm., are cemented to the cover slip squares. The mirrors are made by silvering or aluminizing cover slips and then cutting to size. The "plates," "cups," and mica membranes are sealed together with lubricant (No. 591822/39210—*British Drug Houses Ltd.*). Another form of chamber (Fig. 138) was used by Berenblum, Chain, and Heatley (1940) for the determination of the respiratory quotient of tissue.

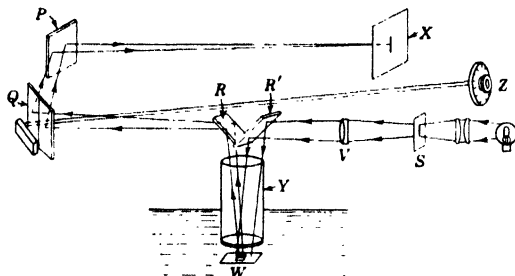



Fig. 141. Schematic diagram of optical system. The image of the split (*S*) is thrown upon the ground-glass screen (*X*) by the mirror system (*R'*, *W*, *R*, *Q*, *P*). If the mirrors *W* are parallel, a single image will result; if the mica bulges, the mirrors *W* will tilt in opposite directions and the image will divide in two. Dial *Z* controls the position of one half of the divided mirror Q. *From Heatley (1940)*

The chamber is mounted in a metal case (Fig. 139) and the cases are fitted into a revolving frame (Fig. 140). The chambers may be opened or closed by rotating the "plate" over the "cup" through 60°. This is accomplished by turning disc *C* of the metal case (Fig. 139). A diagram of the optical system is shown in Figure 141, and the principle of the pressure regulator is illustrated in Figure 142. Finally, a view of the complete apparatus is given in Figure 143, in which the following designations are used:

A, thermobarometer; *B*, supporting bracket for revolving frame; *C*, safety tube to regulate maximum gas pressure; *D*, thermoregulator; *E*, manometer;

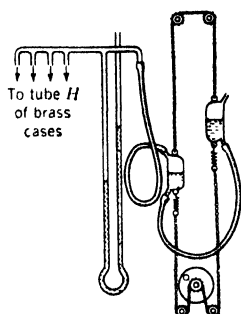



Fig. 142. Principle of manometer and pressure regulator. *From Heatley, Berenblum, and Chain (1939)*

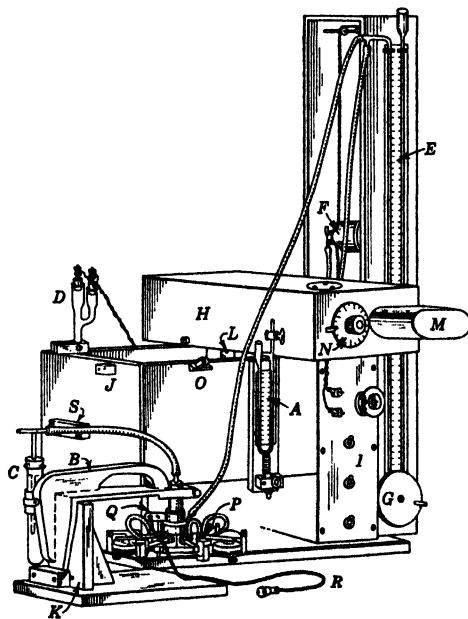



Fig. 143. Diagrammatic view of complete apparatus. *From Heatley (1940)*

F, one of the reservoirs of the pressure-regulating system; *G*, crank-actuating reservoirs of pressure-regulating system; *H*, optical box; *I*, control panel; *J*, guide block for accurately placing frame bracket; *K*, stand for frame bracket when not in use; *L*, brass tube projecting from underside of optical box; *M*, black felt hood protecting ground-glass screen from stray light; *N*, dial operating divided mirror; *O*, pulley over which passes cord for rotating frame; *P*, revolving frame for brass cases; *Q*, ratchet arm for rotating frame; *R*, cord attached to latter; *S*, support for frame bracket during experiment.

The constants of the respiration chambers are determined as for Warburg vessels, mercury being used to measure the total volumes of the chambers.

C. POLAROGRAPHIC

Polarographic methods have been employed for the determination of certain elements in small amounts of tissue, *e.g.*, the procedures of Carruthers for sodium (1943a), magnesium (1943b), and copper (1945), which were used for studies of carcinogenic changes in mouse epidermis. These procedures require several hundred milligrams of tissue for analysis and hence are suited to histochemical work only when a relatively large quantity of histologically well-defined material is available. Because of the limited application these methods will not be given here.

On the other hand, polarography has been applied more readily to respiration studies on the histochemical level. The mercury from the dropping electrode may affect biological systems, but this possible difficulty has been eliminated by Laitinen and Kolthoff (1941a,b), who developed a method for the estimation of oxygen in solution using a platinum wire electrode in place of the mercury type. Contact between mercury and the biological material is also avoided in the double vessel of Selzer and Baumberger (1942). The determination of the oxygen content of body fluids by means of the polarograph was described by Beecher *et al.* (1942), but the method of Davies and Brink (1942) would appear to offer the best opportunity for the application of the polarographic technique to respirometry on a scale suitable for histochemical work. The apparatus of the latter investigators will be discussed in detail.

Microelectrode Measurement of Local Oxygen Tension in Tissue

Davies and Brink (1942) described two types of stationary platinum microelectrodes by means of which local oxygen tensions in

animal tissues can be determined with a spatial resolution of 25 μ. In one electrode the end of a platinum wire is recessed inside a cylindrical glass tip; this instrument may be used to measure absolute oxygen tensions as often as once every 5 min. In the other electrode the end of the wire is directly exposed to the outer medium; with this electrode relative oxygen tensions may be recorded continuously. An idea of the applicability of the method may be gained from the work of Davies and Brink, who employed the electrodes for the measurement of the oxygen tension at the surface of superficial arterioles and venules of the cat cerebral cortex, in the cortical substance, at the surface of muscle cells, and at chosen distances from the surface of unicellular organisms.

The principle of the method is based on the reduction at a platinum electrode of the dissolved oxygen according to the following equation:

$$2\,H^+ + O_2 + 2\,e \rightarrow H_2O_2$$

and the measurement of the currents developed when suitable potentials are applied, the currents being proportional to the oxygen tension.

The variation in electrode current with applied potentials is illustrated in Figure 144. The currents in the plateau region of the

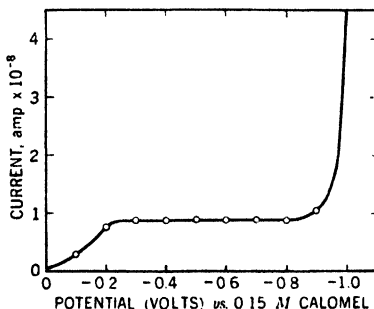



Fig. 144. Current–voltage curve for recessed electrode no. 11 in air-saturated 0.15 *M* NaCl. Temperature, 37°C. Recess length, 1.0 mm. Bore, 0.176 mm. Each point is the value of current 20 sec. after closing the circuit. Re-equilibration time, 20 min. between measurements. *From Davies and Brink (1942)*

curve are limited by the maximum rate at which oxygen can diffuse to the cathode, and this rate is proportional to the oxygen tension. Also, the effect of change in the applied potential is a minimum in the plateau region, and, accordingly, it is in this region that the potentials are chosen for the measurement. The increase in the

current above 0.8 volt is due to a second reaction, *i.e.*, reduction of ionic to molecular hydrogen.

Calibration and Measuring Instruments. The set-up for calibration is illustrated in Figure 145. The platinum electrode (*A*)

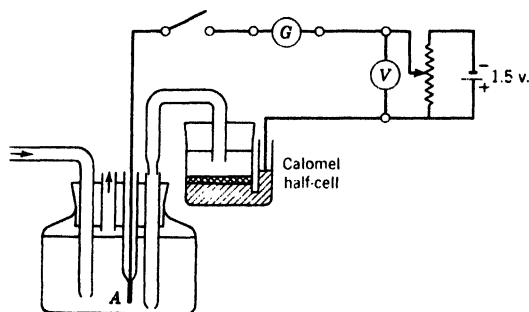



Fig. 145. Diagram of apparatus used for calibrating a stationary platinum electrode.
From Davies and Brink (1942)

dips into a 0.15 *M* sodium chloride solution and mixtures of oxygen and nitrogen may be bubbled through the solution as indicated. The calomel half cell is filled with the same solution and the potential is supplied by a voltage divider. The potential is measured on the voltmeter (*V*) and the current on the galvanometer (*G*).

When the electrode is used on tissue the surrounding tissue fluid serves as the indifferent electrolyte and the calomel half cell is placed as near the electrode as possible, touching either the exposed tissue or a salt pad on another area of the organism.

With an air-saturated solution and electrodes made with wire of 0.2 mm. diameter or larger, currents of the order of 1×10^{-8} amp. or greater are obtained. A galvanometer with a sensitivity of 5×10^{-10} amp. per mm. may be used. With electrodes of smaller diameter, *e.g.*, 25 μ, the currents are of the order of 1×10^{-10} amp. for air-saturated solution and may be measured using a direct-coupled amplifier. In the latter instance the galvanometer is replaced by a well-shielded resistance whose value (*R*) is chosen to effect a potential drop of about 1 millivolt applied to the input of the amplifier. The current (*i*) is given by the expression:

$$i = \Delta E/\gamma R$$

where γ represents the voltage gain of the amplifier and ΔE the change in output voltage resulting from the change iR in input voltage. The grid current of the input tube of the amplifier also passes through resistance R but does not affect the measurement appreciably. The very small currents make it imperative to avoid leaks to the ground, and for this reason the switch and current-measuring instrument are put on the platinum electrode side of the circut. A coaxial cable with polystyrene bead spacers is used as the shielded lead from the electrode and the end of the glass electrode shank is coated with Petrowax (*Gulf Oil Co.*). In this fashion leaks can be held down to the negligible magnitude of 1×10^{-13} amp.

Recessed Electrodes. The recessed electrodes are prepared by sealing a platinum wire in a soft-glass tube of comparable inside diameter in such a fashion that the tube extends beyond the end of the wire to form a recess of the chosen length (recesses of 0.6–1.6 mm. have been used by Davies and Brink). To avoid gas bubbles in the seal it is essential to degas the platinum before the sealing by flaming it to white heat. The recess should be a uniform cylinder whose axis coincides with that of the wire. The completed electrode is annealed at 425° to prevent formation of cracks, which would cause electrical leakages. In the preparation of small electrodes, *e.g.*, with a recess of 0.6 mm. length and 25 μ inside diameter, greater control in the sealing requires the use of electrically heated platinum loops. One loop, 0.3 mm. diameter, is used for sealing the wire into a small glass tube, while another loop, 4 mm. diameter, is used to seal the small tube unit into a larger glass shank. A low-power microscope aids in the observation of the sealing and allows for greater control of the process. The current-potential curves for these small electrodes have more poorly defined plateaus than those obtained with larger electrodes.

In testing, the electrode is placed in the circuit shown in Figure 145, and sufficient time is allowed for the oxygen in the recess to attain equilibrium with that dissolved in the solution. After setting the potential the switch is closed, and the current falls as the concentration gradient spreads into the solution. After a fixed number of sec. from the time the switch was closed the current reading is taken. Then the switch is opened and sufficient time is allowed for the

restoration of equilibrium before another measurement is made. The operation is repeated at other potentials to obtain a current-voltage curve (Fig. 144).

The time the circuit must be left open between readings must be determined for each electrode. This is accomplished by plotting current readings against time between successive closings of the circuit. For each measurement the same number of sec. must be allowed to elapse between the circuit closing and the reading. From Figure 146

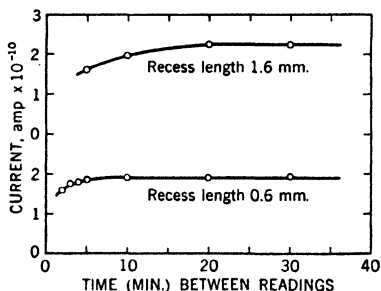



Fig. 146. Twenty-second current as a function of time between readings for two recess lengths. Bore, 25 μ. Air-saturated 0.15 *M* NaCl. Temperature, 37°C. Potential, —0.60 v. *vs.* 0.15 *M* calomel. *From Davies and Brink (1942)*

it is apparent that the readings can be taken every 20 min. with the electrode having the 1.6 mm. recess, and every 10 min. with the 0.6 mm. electrode.

The current, as a function of the time elapsing between closing the switch and taking the reading, can be predicted on the basis of diffusion theory. If the electrode recess is a true cylinder and if readings are taken before the concentration gradient has extended beyond the orifice, the diffusion will be one dimensional and the following relationships will hold:

$$C_{x,t} = C \frac{2}{\sqrt{\pi}} \int^{x/2\sqrt{Dt}} \exp(-y^2)\, dy$$

$$i_t = n\mathrm{F}_y CA(D/\pi t)^{1/2}$$

where $C_{x,t}$ = concentration of oxygen (moles/ml.) at distance x cm. from the platinum surface at t sec. after the start of diffusion (closing the electrode switch), C = initial uniform concentration of oxygen, y = a variable of integration, D = diffusion coefficient of oxygen (cm.²/sec.), i_t = electrode current (amp.) at time, t, n =

no. of electrons used per molecule of oxyge nelectrolyzed, $F_y = 1$ faraday (96,500 coulombs), A = area of a platinum surface (cm.[2]).

From the preceding relationship the current is inversely proportional to the square root of the time (t). When the time is increased to the degree that the gradient extends into the solution outside the recess, the oxygen diffuses to the tip of the electrode from all directions instead of from only one, and this results in a concentration at the orifice higher than that for the linear diffusion. Thus the current is increased beyond that expected from the $1\sqrt{t}$ relation. This is illustrated in Figure 147, which shows the linear relationship

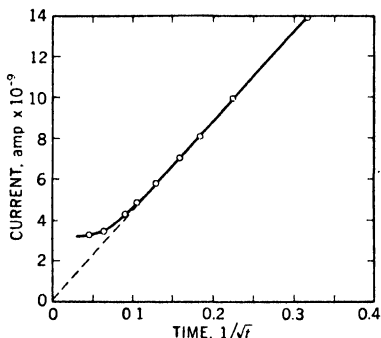



Fig. 147. Current–time curve for electrode no. 11. Air-saturated 0.15 *M* NaCl. Temperature, 25°C. Potential, —0.60 v. *vs.* 0.15 *M* calomel. *From Davies and Brink (1942)*

up to 40 sec. Although the profile of the current-time curve is influenced by the shape of the recess in the electrode, the current at a given time after closing the circuit is still proportional to the oxygen tension as long as there is no change in D. Thus, if the concentration gradient is confined to the recess when the current is measured, only the length of the recess will be important and it will not matter whether the cross-sectional area of the recess is uniform.

The distance from the platinum surface to which the concentration gradient should extend at times after the onset of electrolysis is shown in Figure 148. Up to 20 sec. the gradient extends to 1 mm. from the platinum surface.

In Figure 149, the electrode current at intervals after the start of diffusion is shown as a function of the initial uniform oxygen tension of the solution in recess after equilibration with known gas mixtures. While the electrodes are calibrated at the temperature of the

tissue to be studied, the calibration curves in Figure 149 are actually unchanged within the experimental error between 28° and 37°. The negligible effect of the temperature change results from counterbalancing the associated changes in D and the solubility of oxygen However, the temperature must be known in order to convert partial pressures into concentrations of oxygen.

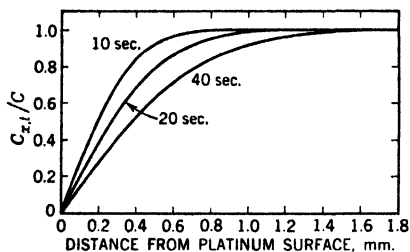



Fig. 148. Concentration–distance curve at various times after beginning linear diffusion. Assumed value of D, 4.0×10^{-5} cm.²/sec. *From Davies and Brink (1942)*

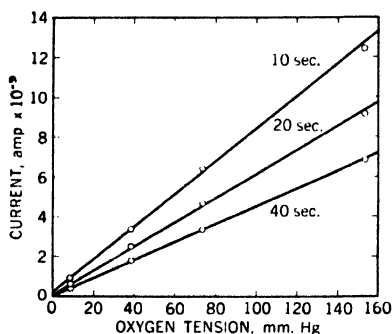



Fig. 149. Current *vs.* oxygen tension from current–time curves. Electrode No. 11. Same solution, temperature, 28°C. Potential, —0.60 v. *vs.* 0.15 *M* calomel. *From Davies and Brink (1942)*

Control experiments have revealed no evidence that substances other than oxygen are electrolyzed under the conditions employed. The recessed electrode offers the distinct advantage that the measurements may be made independent of the diffusion coefficient in the medium beyond the orifice. To maintain constancy of the diffusion coefficient within larger recesses, they are filled with agar gel containing 0.15 *M* sodium chloride. The smaller recesses exclude particulate matter by virtue of the narrow bore; the diffusion of solutes into the recess has no appreciable effect on the value of D. Another advantage of the recessed electrode is its freedom from the effects of convection in the external solution.

When used in blood, the electrode should have its recess filled with agar containing salt to keep the red cells out of it, as these would elicit abnormally high currents by virtue of their function as oxygen sources.

The recessed electrode has been found to give calibration constants which are reproducible to ±3% over a period of weeks. Continued exposure to tissue fluids for some hours will result in a drift in the calibration to yield oxygen tension values which are too low. However, an equal exposure to 0.15 *M* sodium chloride will bring the values back to the correct level. Davies and Brink suggest that this difficulty may be obviated by calibration in the tissue fluids. Later they found that by filling the recess with distilled water and then covering the tip of the electrode with a collodion membrane, the drift in calibration during exposure to tissue fluids can be made very small.

Open Electrodes. The open type of microelectrode is prepared by fusing a platinum wire, 25 μ diameter, into a soft-glass tube so that one end of the wire is flush with the sealed end of the tube. While the open electrode cannot be used for the measurement of absolute oxygen tension, it can serve to measure rapid changes in tension. Thus, Davies and Brink were able to record, to about 0.1 sec., the sudden oxygen consumption occurring when a muscle fiber contracts.

The variable properties of the open electrode make for considerable instability in its performance. However, polarization for several min. at 1.0–1.2 volts effects some stabilization; even so the reproducibility is only about 15% under favorable circumstances. Calibration before and after each experiment will hold the error to a minimum. Poorly defined plateaus are obtained in the current-potential curves, but as a rule with up to 0.8 volt nothing but oxygen is electrolyzed in oxygen-free solution. A linear relation may be found between current and oxygen tension in calibration experiments; however, when applied to tissue, particularly near blood vessels, there may be little correlation between the actual tension and that derived from a calibration curve which is obtained with a solution such as Ringer's.

V. DILATOMETRIC TECHNIQUES

As Sreenivasaya and Bhagvat (1937) pointed out in their review of the subject, dilatometry has seen comparatively little application in the study of chemical and physical changes, although the recognition of its possibilities is by no means new. The adaptation of dilatometry to fine quantitative measurements, with particular reference to histo- and cytochemistry, was made by Linderstrøm-Lang (1937a). The feature of this adaptation is a density gradient in a nonaqueous medium in which a very small drop of aqueous reaction mixture is suspended. Changes in volume of the drop that accompany the chemical changes taking place within it are made manifest by a vertical displacement to a new position where the specific gravities of the drop and the surrounding medium are again equal. The magnitude of the displacement then becomes a measure of the extent of the reaction that occurred within the drop. Standardization of the density gradient is accomplished by introducing aqueous drops of known densities.

The method is obviously limited to those systems that do not contain or evolve constituents soluble in the bromobenzene-kerosene medium. Thus lipase measurements could not be made in this manner. The sensitivity of the method largely depends on the magnitude of the contraction constant, which may be defined as the volume change occurring when one gram molecule of reactant undergoes chemical change. Expressed mathematically,

$$K = vM/cV$$

where K is the contraction constant, M the molecular weight, v the change in volume, V the original volume, and c the concentration. The constant for the hydrolysis of urea is 24.1; hence, when 60 g. (1 mole) urea is hydrolyzed, the reaction mixture decreases in volume by 24.1 ml. It is important that the temperature be held very constant during the measurement so that the change determined is only the isothermal one. The advantage of this method lies, not only in the circumstance that very small reaction volumes may be

employed, but also in the fortunate fact that the measurements may be made without disturbing the system in any way.

DILATOMETRIC APPARATUS AND ITS USE

Gradient Tube. The glass gradient tube, that is employed to furnish a practically linear specific gravity gradient, has the form and dimensions shown in Figure 150. The tube is mounted in a thermostat that maintains its temperature constant to about ±0.002°; the group at the Carlsberg Laboratory have done their work at 30°. The tube is first filled with bromobenzene (sp. gr. 1.48)

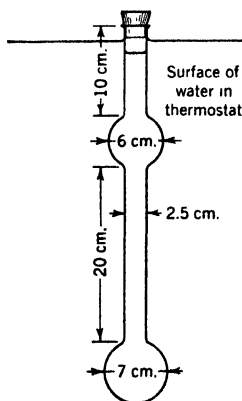



Fig. 150. The gradient tube. *From Linderstrøm-Lang and Lanz (1938)*

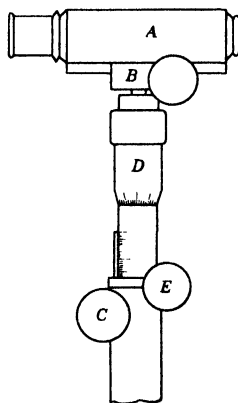



Fig. 151. Measuring microscope. *From Linderstrøm-Lang and Lanz (1938)*

up to the middle of the part connecting the two bulbs. After the bromobenzene has attained the temperature of the bath, "water-white" kerosene (sp. gr. 0.79) having the same temperature is added carefully through a funnel fitted with filter paper to fill the remainder of the tube. The surface of the liquid in the tube should be below that of the thermostat water. A long spatula is used to agitate gently, for about 10 sec., the liquids in the region of their junction in the middle of the tube so that some mixing will occur. After 24–48 hr. the density gradient is sufficiently linear. Usually the gradient is maintained for months and even years.

Saturation of Medium with Water. It is necessary to saturate the medium with water at a suitable vapor pressure in order to minimize the tendency of the aqueous drop to lose water to the medium and thus change its specific gravity. It has been found sufficient to employ a 0.2 *M* potassium bromide solution for the range of specific gravity from 0.99–1.01. About 1 ml. of the salt solution is shaken thoroughly with about 10 ml. of the less dense kerosene-bromobenzene mixture. The resulting suspension is poured at once into the gradient tube, and as the drops fall they saturate the medium. The high specific gravity of the drops carries them down far enough in the tube to avoid any interference with subsequent measurements.

Gradient Calibration. Calibration of the gradient is accomplished by the simple expedient of placing in the tube small drops (0.10–0.15 μl.) of potassium chloride solutions of known specific gravities (Table VIII). It is well to store these standard solutions under a 1 cm. layer of kerosene in stoppered vessels, such as 50 ml. volumetric flasks, which restrict the surface exposed to the kerosene. In some cases, such as in the determination of "reduced weight", (page 420), standard drops composed of mixtures of doubly distilled water and deuterium oxide have been used. The 0.10–0.15 μl. pipette used to add the drops may be either the type shown in Figure 51

TABLE VIII

Composition and Density[a] of Standards for Dilatometry

Standard No.	Potassium chloride, %	d_4^{30}	Standard No.	Potassium chloride, %	d_4^{30}
0	0	0.995673	6	1.0272	1.002155
1	0.1719	0.996785	7	1.1848	1.003149
2	0.3408	0.997823	8	1.3442	1.004155
3	0.5122	0.998905	9	1.5121	1.005214
4	0.6633	0.999857	10	1.6904	1.006339
5	0.8450	1.001005	11	1.8721	1.007486

[a] The densities are taken from the Landolt-Bornstein tables.

(page 173) or the constriction pipette (Fig. 53, page 173). These pipettes may be calibrated by measuring out strong acid of known concentration and titrating it with a microburette, but for the present purpose it is sufficient to calibrate them more roughly by weighing the quantity of water they deliver directly on the pan of a microbalance.

Method of Adding Drops. The procedure for adding the drops is as follows:

1. Dip the tip of the pipette below the kerosene layer into the solution.
2. Rinse the pipette by drawing up and blowing out the solution several times.
3. Draw the solution to the mark on the pipette.
4. Raise the pipette until the tip is in the kerosene.
5. Draw up about 0.1 μl. kerosene into the pipette.
6. Remove the pipette and wipe the outside with the edge of a piece of filter paper.
7. Blow out about half the kerosene while the tip is touching a piece of filter paper.
8. Dip the pipette into the gradient tube so that the tip is about 2 mm. below the surface of the medium.
9. Blow out the pipette charge.
10. Remove the drop from the tip by lifting the latter out of the medium. The drop falls and reaches its equilibrium position usually within 15 min.

Method of Removing Drops. Drops may be removed in the gradient tube by inserting a long thin glass rod with a fine point to which the drops will adhere. They may then be carried up and placed on the wall of the tube near the surface of the medium. It is impossible to remove them entirely from the medium in this fashion since the surface forces act to detach the drops from the glass tip when this is attempted. The glass rod should be handled carefully to avoid undue disturbance of the density gradient.

Dr. Oliver H. Lowry instituted the practice of adding a little sand (60–80 mesh) from a salt shaker to remove the droplets. This is an effective method which disturbs the gradient less than the glass rod procedure.

Measurement of Drop Position. The position of drops is determined by a horizontal microscope of long focal length mounted on a micrometer stand (Fig. 151). The microscope (A) is pivoted at B; C is a calibrated rough adjustment rack with a range of 100–150 mm., and E is a clamp to hold the rack at any given setting. The micrometer screw D enables fine adjustment, it has a vertical

range of 10–12 mm. with one complete revolution giving a movement of 1 mm. Since the circumference is divided into 100 parts, measurements may be made with an accuracy of 0.01–0.02 mm. The apparatus is mounted on a triangular base fitted with leveling screws.

The position of a drop with reference to a standard drop is determined by turning the micrometer screw to zero, setting the reference mark in the microscope ocular (cross hair or scale division) at the lower edge of the standard drop by means of rack *C* and clamp *E*, moving the microscope horizontally so that it will be in line with the drop to be observed, and bringing the reference mark to the lower edge of the latter drop by adjustment of the micrometer screw.

Measurement of Reaction Rate. When the gradient tube contains a series of standard drops embracing the range of specific gravity within which the reaction mixture falls, measurements of reaction rate may be carried out by determination of positions at suitable intervals. Readings are begun after 15–20 min. from the time the drop of reaction mixture is introduced into the tube. It has been shown that this is an adequate time to allow the drop to assume its proper position for observation. A plot of the densities of the drop as a function of time represents the course of the reaction.

PEPTIDASE

The use of the gradient tube for dilatometric determination of peptidase activity was described by Linderstrøm-Lang and Lanz (1938). They employed DL-alanylglycine as substrate, of which only the D form is hydrolyzed enzymatically by pig stomach and intestinal extracts, which were used as the source of the enzyme. The change of density with time was found to be a linear function. The contraction constant for the scission of the peptide bond was found to vary with both *p*H and the concentration of the phosphate buffer employed. The magnitude of this variation, as determined by both direct macro dilatometry and the micro method, is shown in Table IX. These measurements were made at 30° on reaction mixtures consisting of equal volumes of substrate and enzyme solutions. The substrate solutions had the composition given in Table X; the *p*H values were calculated on the assumption that the *p*K value for DL-alanylglycine at 30° is 8.09. The enzyme solutions were prepared

by grinding 30 g. pig intestine with sand and 100 ml. 60% glycerol; after filtering, 1:50 dilutions were made with phosphate buffers having the different *p*H values and concentrations indicated in the table. Controls were run in which the enzyme solution had been heated at 100° for 30 min. in a sealed glass tube.

TABLE IX

Contraction Constants for Alanylglycine Hydrolysis by Peptidase from Pig Intestine at Different *p*H Values and Phosphate Concentrations[a]

Phosphate concn. in reaction mixt.	*p*H 6.8		*p*H 7.1	*p*H 7.4		*p*H 7.7	*p*H 8.0	
	Micro method	Direct dilat.	Micro method	Micro method	Direct dilat.	Micro method	Micro method	Direct dilat.
M/60	9.1_6	9.1_8	9.3_5	9.4_7	9.4_4	9.9_9	11.4_2	9.4_1
M/120	9.1_2	—	—	9.2_8	9.1_2	—	11.2_2	—
M/240	—	—	—	9.2_2	—	—	—	—

[a] μl./m*M* peptide bond.

TABLE X

Composition of Substrate for Peptidase Measurements at Various *p*H Values

*p*H	Molarity of DL-alanylglycine	Molarity of NaOH
6.81	0.2	0.0100
7.13	0.2	0.0193
7.42	0.2	0.0346
7.72	0.2	0.0611
8.00	0.2	0.0900

The deviations in the constants as determined by both methods at *p*H 7.7 and 8.0 are not understood. Accordingly, Linderstrøm-Lang and Lanz have chosen to limit the drop method, for the time being, to reaction mixtures with *p*H values in the range 6.8 to 7.4.

Enzyme experiments lasting 24 hours may be safely carried out. The accuracy of the method is about fifty times that attained by the microtitration procedure (page 302). An idea of the quantity of material that may be subjected to investigation by the density gradient method is given by calculations included in the paper of Linderstrøm-Lang and Lanz. They estimated that if 10 hours be taken

as a maximal time for measurements, and the density determined to 1×10^{-5}, with 0.1 μl. drops, the least quantity of alanylglycine splitting that could be detected in 10 hours would be 1×10^{-7} millimole. This splitting could be accomplished by one thousandth of one sea urchin egg, corresponding to about 5×10^{-8} mg. dry organic material.

Jacobsen (1942) showed that the contractions accompanying the cleavages of peptide linkages in benzoyl-DL-argininamide by trypsin and benzoyl-L-tyrosylglycinamide by chymotrypsin are 13.4 ± 0.2 and 15.6 ± 0.8 ml. per mole, respectively.

Method of Linderstrøm-Lang and Lanz for Peptidase

SPECIAL REAGENTS

Enzyme Preparation. 60% glycerol extract of tissue diluted with phosphate buffer, *p*H 6.8 to 7.4, or a bit of cellular material can be used directly.

Substrate Solution. 0.2 *M* DL-alanylglycine containing sodium hydroxide of the molarity indicated in Table X to give the desired *p*H.

Mixture for Saturation of Medium. Combine equal vol. of 0.2 *M* potassium bromide and substrate soln.

PROCEDURE

1. Saturate the medium in the gradient tube with the potassium bromide-substrate soln. mixture in the manner described in the preceding section. After 24 hr. the tube may be used.

2. Introduce a number of 0.15 μl. standard drops into the tube. The sp. gr. of these drops should bracket the range encountered in the determination. Two drops of the same standard should not deviate from one another in their equilibrium levels by more than 0.1 mm.

3. Mix equal vol. enzyme and substrate soln. under kerosene in a small test tube, and place 2–3 drops 0.15 μl. each in the tube. For microtome sections of tissue, or smaller cellular units, first place a 1 μl kerosene drop on the end of a glass needle about 0.2 mm. thick; insert the tissue into this drop with the aid of a very fine glass needle, dip the drop containing the tissue into the gradient tube

medium, and slowly raise the tip of the needle out of the liquid to disengage the drop.

4. Repeat preceding step with control drop containing heat-inactivated enzyme.

5. Record positions of drops, relative to standard drops, at intervals of about 15 min., until the rate of change in position of both active and control drops is equal, indicating that the enzymatic process is complete.

6. Plot results to determine the slope relating density change with time. When the size of the drops and experimental conditions are kept constant, the millimoles of alanylglycine split per unit time, R, is given by the equation:

$$R = (S\nu/Kd)$$

where S represents the slope, ν the drop vol., K the contraction constant under the experimental conditions, and d the drop density.

DENSITY AND "REDUCED WEIGHT"

Aside from its use to follow the course of reactions, the density gradient technique can be employed as a very convenient method for the measurement of the density of very small amounts of material. Linderstrøm-Lang, Jacobsen, and Johansen (1938) applied the technique to the measurement of the deuterium content in mixtures of water and deuterium oxide. Jacobsen and Linderstrøm-Lang (1940) modified the apparatus for more rapid determinations of specific gravity by the use of a 200 ml. graduated cylinder as a gradient tube, omitting thermostatic control and the use of the traveling microscope. This simplification is well adapted to the measurement of the specific gravity of biological fluids with an accuracy of 0.1%. Lowry and Hunter (1945) employed a similar apparatus for the determination of serum protein concentration.

It is of particular interest that the gradient tube has been adapted to the determination of "reduced weight" (Linderstrøm-Lang and Holter, 1940), since the latter value affords a measure of the size of a tissue sample independent of its water content. In some respects this circumvents one of the chief difficulties encountered in histochemical and cytochemical investigations, *i.e.*, obtaining a quantitative definition of structural elements in tissues or cells to which quantities of a

constituent measured can be referred. The "reduced weight" is the weight of the sample minus the weight of an equal volume of water, or expressed mathematically:

$$g_r = g - (v_g \, d_w)$$

where g_r is the "reduced weight," g the weight of the sample, v_g the volume of the sample, and d_w the density of water at the experimental temperature. When the gradient tube is employed to determine the "reduced weight," it is only necessary to place the sample in a small drop of water, measure the specific gravity of the unit as a whole, obtain the diameter of the water drop with the aid of the micrometer microscope and from this value calculate the volume, and finally apply the equation:

$$g_r = (d - d_w)v$$

where d is the specific gravity of the drop containing the sample, and v is the volume of this drop. For standardization of the gradient, drops are used consisting of mixtures of double-distilled water and deuterium oxide; and they have density differences of about 1×10^{-3}.

The accuracy of the method depends on the size of the drop. For 0.1 μl. drops it is about 5×10^{-7} mg. and for 1.0 μl. drops it is about 5×10^{-6} mg.

The tissue sample employed for "reduced weight" measurement can be used subsequently for enzymatic determination, provided the kerosene-bromobenzol medium has no influence on the activity. The drop containing the tissue sample is removed from the gradient tube by means of a retriever consisting of a thin glass rod the tip of which is fused to a piece of cover glass, 2 $\times$ 2 mm., at an angle a little greater than 90°. Once removed from the tube, the material can be transferred to the medium appropriate for the enzyme measurement.

VI. DETERMINATION OF AMOUNT OF A BIOLOGICAL SAMPLE

A major problem in histo- and cytochemistry is the quantitative definition of the samples of biological material which are to be considered. Weight, volume, numbers of cells, and nitrogen or nucleic acid content have been variously employed for this purpose, and both advantages and disadvantages are to be found in each case. The actual choice of a reference quantity will depend on the particular problem to be considered.

Weight. Wet- and dry-weight measurements may be made with commercial microbalances including torsion balances such as those manufactured by *Roller-Smith Co.,* which are sensitive down to about 2 μg. Quartz fiber balances of greater sensitivity are described on page 189.

It is advantageous in some instances to employ the so-called "reduced weight" which is the weight of the sample minus the weight of an equal volume of water. The feature of this value is that it is independent of the water content of the sample. "Reduced weight" can be determined rather simply in the gradient tube apparatus by placing the sample in a drop of water and measuring the density of the drop. From the density, the volume of the drop, and the density of water, the "reduced weight" can be calculated (page 421).

Volume. When the sample, such as an *Amoeba proteus,* is of a nature and size that permits it to be drawn into a capillary tube of known diameter, the volume can be calculated after the length of the sample in the tube is measured. However, this procedure cannot be used if the sample has an irregular shape which does not allow it to completely fill the lumen of the capillary over the entire length of the body or if it is so sticky that it adheres to the wall of the capillary and thus interferes with the manipulation or becomes damaged. To determine volumes in these cases, Holter (1945) developed a colorimetric method which he applied to measurements of the amoeba, *Chaos chaos.* The details of the procedure are given on page 432.

The volume of microtome sections of tissue is regulated by cut-

ting circular sections of a known thickness and diameter as described on page 427.

Numbers of Cells. When the cells are in suspension their number may be determined by counting in a hemocytometer chamber. It may aid in the counting to stain the cells first. However, the measurement of the number of cells in histological preparations is more involved. A technique for cell counting in mounted stained sections of tissue was described by Linderstrøm-Lang, Holter, and Søeborg Ohlsen (1934).

The data of Rask-Nielsen (1944) for the number of cells per unit volume of pyloric mucosa may be taken to illustrate the counting technique. A microscope magnification of 300× was used, and the ocular was equipped with an ocular micrometer containing a circle, divided into quadrants, which enclosed an area corresponding to 0.0638 $mm.^2$ of the tissue section. Six to ten random counts, uniformly distributed over the stained section, were made of all nuclei and fragments thereof within the bounds of the counting area.

In a given case a mean of the chief cells from eight random counts was 229. The mean error of the mean value of the counts was ±4.4, obtained from the formula:

$$\sqrt{\frac{\Sigma\Delta^2}{q(q-1)}}\sqrt{1-\frac{q\cdot a}{A}}$$

where($\Sigma\Delta^2$) is the sum of the squares of the individual deviations from the mean value, q the number of counts, a the counting area, and A the area of the section.

In order to convert the number of nuclei plus fragments per counting area (229 ± 4.4) to cells per counting area, a correction factor for the fragments must be applied. This factor is $12/(12 + h)$ where 12 is the thickness of the section and h the height of the nuclei, both expressed in microns. In this case the factor equals 0.71; hence:

$$(229 \pm 4.4)\ 0.71 = 163 \pm 3.1 \text{ cells/counting area}$$

Since the sections were cut from a cylinder of fresh-frozen tissue having a diameter of 2.64 mm.:

$$\frac{\pi}{4}(2.64)^2 = 5.47 \text{ mm.}^2\text{, area of a fresh section}$$

$$5.47 \times 0.012 = 0.066\ \mu\text{l., volume of a fresh section}$$

and

$$\frac{5.47}{0.0638} = 85.7 \text{ counting areas/fresh section}$$

However, the fresh-frozen sections were fixed and stained before the counting so that a correction is required to compensate for the contraction produced by this treatment. Since the counting was carried out through the entire thickness of the section, the contraction in thickness need not be considered because the ratio of the actual number of cells to the cells counted will not be altered by a change in thickness. The change in area must be considered, however. The ratio of the area of each stained section to that of the fresh section (5.47 mm.^{2}) was obtained by projecting both images at the same magnification and measuring the respective areas with a planimeter. The areas might also be obtained by tracing the outlines of the projected images on paper and weighing the paper. Then the actual number of cells per section is

$$\begin{pmatrix}\text{No. stained cells} \\ \text{per counting area}\end{pmatrix} \times \begin{pmatrix}\text{no. counting areas} \\ \text{per fresh section}\end{pmatrix} \times \left(\frac{\text{area of stained section}}{\text{area of fresh section}}\right)$$

And this quantity divided by the volume of a fresh section equals the actual number of cells per unit volume of fresh tissue.

In the present case:

$$\frac{(163 \pm 3.1)\ (85.7)\left(\frac{5.08}{5.47}\right)}{0.066} = (196 \pm 3.7)\ 10^3 \text{ cells}/\mu\text{l}.$$

In regions of inhomogeneity within the section the relative areas of the mutually deviating regions were measured after projecting the images on paper, and the separate regions were counted. Each of the regions of a given cell type was made to contribute to the final mean result as illustrated in the following examples from Rask-Nielsen (1944):

(1) *In the case of a section containing one type of cell not homogeneously distributed*:

In 61% of the section area the cells were arranged in glandular tubules. Mean of five random counts was 306. Mean error ±6.3.

In 39% of the section area the cells were arranged in pits. Mean of four random counts was 203. Mean error ±14.8. Mean number of nuclei plus fragments per counting area was:

$$(306 \pm 6.3)\ 0.61 + (203 \pm 14.8)\ 0.39 = (187 \pm 3.8) + (79 \pm 5.8) = 266 \pm 9.6$$

Applying the correction (0.71) for nuclei fragments:

$$(266 \pm 9.6)\ 0.71 = 189 \pm 6.8 \text{ cells/counting area}$$

and then following the formula used previously:

$$\frac{(189 \pm 6.8)\ (85.7)\ \left(\frac{5.30}{5.47}\right)}{0.066} = (238 \pm 8.6)\ 10^3 \text{ cells}/\mu\text{l}.$$

(2) *In the case of two different types of cells in the same section*:

Epithelial cells comprise 25% of the area of a section.
Neck chief cells comprise 75% of the area of the section.
Epithelial cell count = 262 ± 6.7.
Epithelial cells per counting area = (262 ± 6.7) 0.71 = 186 ± 4.8.

$$\frac{(186 \pm 4.8\ (85.7)\ \left(\frac{5.00}{5.47}\right)}{0.066} \times 0.25 = (55 \pm 0.14)\ 10^3 \text{ epithelial cells}/\mu\text{l}$$

Neck chief cell count = 326 ± 8.5.
Neck chief cell per counting area = (326 ± 8.5) 0.71 = 232 ± 6.0.

$$\frac{(232 \pm 6.0)\ (85.7)\ \left(\frac{5.00}{5.47}\right)}{0.066} \times 0.75 = (206 \pm 5.3)\ 10^3 \text{ neck chief cells}/\mu\text{l}.$$

The determination of a constituent in each type of cell in this section may follow the example given:

From the nearest section containing only epithelial cells a peptidase activity of 0.125×10^{-3} units per cell was found. Therefore, the activity arising from the epithelial cells was:

$$(55 \pm 0.14)\ 10^3 \times (0.125)\ 10^{-3} = 6.8 \text{ units}/\mu\text{l}.$$

and

$$(6.8)\ (0.066) = 0.45 \text{ unit/section}.$$

The activity of the section composed of both the epithelial and neck chief cells was found to be 1.70 units, of which 0.45 unit was ascribed to the epithelial cells present. Therefore, the activity of the neck chief cells in the section was considered 1.70 — 0.45 = 1.25 units, and the activity of these cells was 1.25/0.066 = 19 units/μl., or $19/206 \times 10^3 = 0.092 \times 10^{-3}$ units/neck chief cell.

Nitrogen. The total nitrogen in a sample has often been used as an indication of the quantity of protoplasm present. Measurements of the nitrogen content may be carried out by one of the procedures given on pages 230–239, and 283.

Nucleic Acid. Berenblum, Chain, and Heatley (1939) employed the estimation of nucleic acid phosphorus as an indication of the quantity of cellular material present, in an attempt to refer measurements to the amount of metabolizing substance rather than to

the total tissue, which would include inactive material. Berenblum *et al.* first removed lipid phosphorus by extracting with alcohol-chloroform (3:1). Organic and inorganic acid-soluble phosphorus was removed by extracting with 0.1 *N* hydrochloric acid; the tissue was then ashed with perchloric acid, and the phosphorus in the ash was measured.

A. PREPARATION OF FROZEN TISSUE SECTIONS OF ACCURATE THICKNESS

In some instances it is undesirable to subject tissue to the embedding process and the associated treatments with the various solvents prior to the application of histochemical tests or analyses on microtome sections. When this is the case, the freezing microtome may be used for either fresh tissue or that fixed in a suitable manner. It may be necessary to obtain sections of accurately uniform volumes for quantitative work. The cross-sectional area of sections can be controlled by punching out cylinders of tissue from frozen material with metal borers of known internal diameters (Fig. 152).

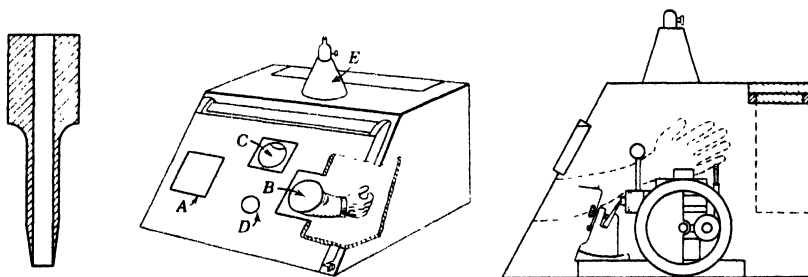



Fig. 152. Tissue borer and microtome cryostat.
From Linderstrøm-Lang and Holter (1940)

The cylinder of tissue can be placed on some wet filter paper set on the freezing head of the microtome and then the whole firmly frozen to the head. This is the procedure that has been followed in many cases by the Carlsberg Laboratory investigators. They have found too that a rotary microtome is less subject to factors which lead to variations in thickness than the hand sliding type, and that the

greatest uniformity is achieved by continuous sectioning at constant speed. The first section is always discarded, and, in order to minimize the temperature effect of the intermittent cooling with carbon dioxide as commonly practiced, it is particularly important to discard the first section after cooling and proceed at once to cutting before appreciable warming can occur. These difficulties have been surmounted to a great degree by Linderstrøm-Lang and Mogensen (1938), who devised a means of maintaining the entire microtome at a constant temperature low enough to keep the tissue frozen on the block, at the same time making possible the free manipulation of the instrument and the sections. In addition they developed a method to prevent undue distortion or curling of sections on the knife edge.

Linderstrøm-Lang and Mogensen Method for Accurate Cutting and Special Handling of Frozen Tissue Sections

A cryostat large enough to hold a rotary microtome is arranged to maintain a constant temperature of around −20°. The type developed by Linderstrøm-Lang and Mogensen (1938) is indicated by Fig. 152. The cabinet is made of two layers of wood, an inner one of 22 mm. furniture board and an outer one of 10 mm. cross cut veneer. A sloping lid is hinged to the front of the cabinet, and through two openings (*A* and *B*), lambskin-lined leather gloves are attached. The gloves are of a size to permit easy manipulation within the cabinet. An observation window (*C*) consists of a flat glass cell filled with water, which is prevented from freezing by a small low-heat electric resistance coil fitted against it. An extra hole (*D*), which is kept closed with a stopper, enables removal of sections from the cabinet. The interior is illuminated by a lamp (*E*) which fits over a glass window in the top. A wooden partition separates the interior into a front and rear chamber, the latter being about half the size of the former. The rear compartment, fitted with a well-insulated lid, is arranged to hold dry ice. Two guide rails fixed on the floor of the front compartment serve to hold the microtome in position, and in order to prevent the frosting of the microtome, an electric heater capable of holding the microtome at 5° in placed between the rails. A thermoregulator fits through the left side of the roof of the micro-

tome chamber, and in the right side of this compartment an electric blower, such as a hair dryer, is so placed that it blows air from the compartment against a movable metal valve that permits the air either to go back directly into the microtome chamber or to pass through the Dry-Ice box before returning. The valve is controlled by the thermoregulator.

Fig. 153. Device for prevention of curling of sections. *From Linderstrøm-Lang and Mogensen (1938)*

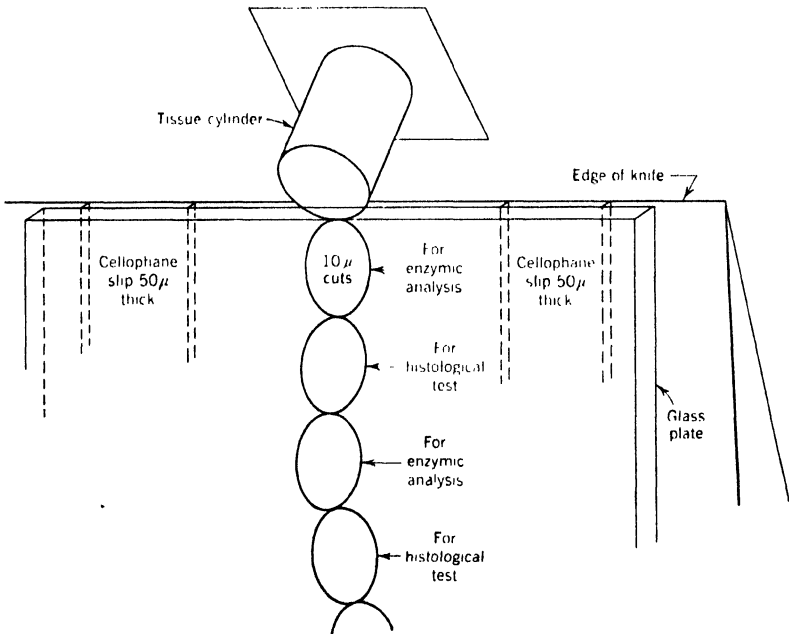
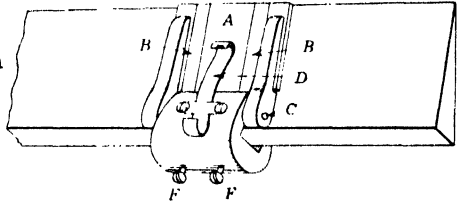



Fig. 154. Use of device for prevention of section curling. *From Linderstrøm-Lang (1939)*

In practice, the microtome without its tissue freezing block is placed in the cabinet, the Dry-Ice chamber is filled with small pieces, the blower is started, and after about 45 min. the temperature is at —20 ±0.5°. A cylinder of tissue is frozen to the block using carbon dioxide, and the block is then brought inside the cabinet through a removable window (*C*). After the block is fastened to the microtome, the knife is set and cutting is begun. When not in use, the microtome is stored in a large desiccator with sulfuric acid.

The sections are prevented from curling on the knife edge by the arrangement shown in Figures 153 and 154. A plate of glass (*A*) is held at a distance of 50 μ from the knife by two strips of cellophane tape (*B*). The glass plate can be moved on the hinge (*C*), and the spring (*D*) holds the plate against the cellophane strips. The upper edge of the plate coincides with the knife edge; screws (*F*) assist in the adjustment. The microtome is operated at constant speed and without stopping for each series of sections to obtain uniform cutting. After the glass plate is swung back, the first section in each series is discarded since its thickness is different from that of the others, and the sections to be used are transferred to slides by a thin glass rod or fine brush.

B. MICROSCOPIC EXAMINATION AND CHEMICAL ANALYSIS OF THE SAME TISSUE SECTION

In the usual procedure, alternate sections are employed for chemical determination and histological examination in order to correlate the analysis with the morphological constitution of the tissue. This procedure is suitable, provided the histology of the section analyzed and that of the adjacent section studied microscopically are essentially the same. When this is not true, as in the case of retinal tissue which has histological changes every 40 to 50 μ, it is necessary to carry out the analysis and the microscopy on the same section. A method for accomplishing this was worked out by Anfinsen *et al.* (1942), who first stained and examined the section and then used it for chemical measurement.

Method of Anfinsen *et al.* for Microscopy and Analysis on the Same Tissue Section

In the procedure of Anfinsen *et al.*, frozen sections are cut in the cryostat of Linderstrøm-Lang and Mogensen, and the sections are allowed to stand in the cryostat until dry. Faster drying can be effected by the use of a dehydrating agent, with or without vacuum. (With phosphorus pentoxide as the desiccant, 20 μ sections of retina were completely dehydrated within 1–1.5 hr. at −20° at atmospheric pressure, or within 15–20 min. *in vacuo.)* A nonaqueous solvent is then used for mild staining to minimize displacement or solution of tissue constituents. (A mixture of 1 vol. of 40 milligram per cent methyl violet in absolute alcohol to 50 vol. xylol was used.) The stained section is washed with xylol, transferred to a slide, and flattened with a cover slip for visual examination or photomicrography. After this the cover slip is removed, excess xylol is absorbed on filter paper, and the section is allowed to dry in the air. Then the dry section may be employed for chemical determination. The preceding treatment was found to have no significant effect on the peptidase or diphosphopyridine nucleotide in rat liver or the cholinesterase in rat brain. In some instances it should be possible to examine the sections directly without staining.

C. VOLUME OF IRREGULARLY SHAPED, SMALL BIOLOGICAL SAMPLES

The measurement of volume as a means of defining the quantity of a biological sample has already been discussed (page 423). Holter (1945) developed a colorimetric method for measuring the volume of irregular objects of the order of 0.01 to 1.0 μl. such as the amoeba *Chaos chaos*. The simple method of drawing an amoeba into a capillary of known diameter, measuring its length, and computing its volume, cannot be used with irregular organisms which do not fill the lumen of the capillary.

In Holter's method, the object is drawn into a capillary tube of known diameter which is wide enough to avoid deformation of the object, and some dye solution of known concentration is taken into the capillary with it. The total length of the object plus dye is meas-

ured and the dye is emptied into a known volume of water. The concentration of dye is determined colorimetrically in a microcuvette and from it the volume of dye that was in the capillary is obtained. This volume is subtracted from the volume of the dye plus object to give the volume of the object. In the range 0.01 to 0.1 μl., objects were measured with a probable error of about ±5% in single measurements.

Holter Method for Measurement of Volume

SPECIAL APPARATUS

Capillaries. Choose capillaries which have a cylindrical constant bore for at least 10 mm. from one end and which have an inside diameter suitable for the object to be measured (0.3 mm. for *Chaos chaos*). Draw out the uncalibrated end of the capillary to a fine tip and mount as a braking pipette (Fig. 123). Fire-polish the wide end of the capillary but do not constrict the mouth. Place a mark about 5 mm. from the mouth; a thread of DeKhotinsky cement may be used. Dry the capillary with alcohol and air between measurements.

Moist Chamber. To prevent evaporation of the small volumes of solution used, manipulations must be carried out in a moist chamber (page 181).

SPECIAL REAGENTS

Dye Solution. Dialyze a soln. of acid violet (sodium tetraethyl-di-*p*-sulfobenzyl-*p,p'*-diaminofuchsonimonium) in glass-distilled water, and bring to a concentration of 1%. Add sodium taurocholate to 0.01% and filter through a fiber-free material. Store in a refrigerator, and about once every two weeks refilter to remove dust and dye crystals which may have formed.

PROCEDURE

1. Transfer the object into a moist chamber by means of a braking pipette (page 359).
2. Place the object in a flat drop of the dye soln. contained in a dish.
3. Wash the object with the dye soln. by drawing it up into the pipette a few times.

4. Transfer the object to a fresh flat drop of the dye soln.

5. Draw the object up into the capillary along with an amount of dye soln. not greater than twice the vol. of the object.

6. Remove the end of the capillary from the drop and draw the object in the dye soln. about 2 mm. in from the mouth.

7. Dip the mouth of the capillary into water and draw in a column about 1 mm. long. Remove from the moist chamber.

8. Place a very small drop of mercury (which will fill 0.5–1 mm. of the capillary) on a watch glass and carefully suck it halfway into the mouth of the capillary and then push it all the way in with the finger. The capillary should now look like the illustration in Figure 155.

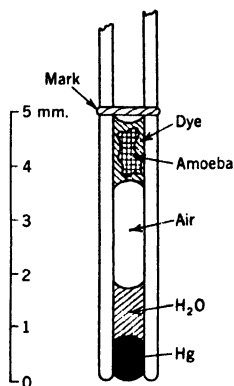



Fig. 155. Capillary for volume measurement, filled.
From Holter (1945)

9. With the capillary in a vertical position, measure the distance between the menisci of the dye soln. with a micrometer miscroscope (Fig. 151) to 0.01 mm. With a living amoeba, it is necessary to use a filter to remove the heat from the source of illumination so that the organism will not be disturbed. Movement of the amoeba to a meniscus may distort, or obstruct the view of the meniscus.

10. Clean the capillary on the outside with wet filter paper, and remove the mercury gently by combining it with a large drop of mercury.

11. Remove the water from the capillary with a tip of a strip of hard moistened filter paper.

12. Empty the dye soln. plus amoeba into a small reaction tube containing a measured vol. water (100 μl.). Rinse the capillary with the water and stir the soln. by means of a magnetic "flea" (page 179). Use a permanent magnet, rather than the periodic electromagnet, to stir carefully to avoid damage to the organism.

13. Remove the "flea" with the magnet, and the object with a braking pipette.

14. Transfer the diluted dye soln. to a cuvette and obtain its colorimeter reading using a yellow filter (S57 with the Zeiss step photometer).

15. Run an empirical set of standards with different lengths of dye columns in the capillary and plot a curve of the colorimeter readings against the lengths of the dye columns. The standard curve may be used as long as neither the capillary nor dye soln. is changed. To check the constancy of the dye soln., run one or two samples of dye alone, which have known lengths in the capillary, in each experiment.

16. Calculate the vol. of the object (V) by the formula:

$$V = A(L_t - L)$$

where A is the cross-sectional area of the capillary, L_t the length of the dye plus object in the capillary, and L the length of the dye alone. The value of L, corresponding to the colorimeter reading of the unknown, is obtained from the standard curve.

VII. DEDUCTIVE METHODS

In certain instances deductive methods can be applied to obtain histochemical data from macrochemical analyses, *i.e.*, if the total amount of a constituent in a tissue and the amount in the entire extracellular portion is known, one can calculate the amount in the intracellular fraction. This method has been exploited by Lowry (1943) and the principle may be illustrated by one of his examples:

"As a first approximation, a tissue such as skeletal muscle may be considered to be composed of 5 separate fractions, blood, fat, collagen plus elastin, extracellular fluid, and cells. If the amount of the first 4 fractions can be determined, the amount of the remaining intracellular fraction can be calculated. Furthermore, if one knows the composition of the blood and extracellular fluid, it becomes possible to calculate the concentration of a particular substance, A, in the cells by simply (1) measuring the total amount of A, (2) calculating the amount of A in the several extracellular fractions, (3) subtracting the extracellular A from the whole, and finally, (4) dividing the net intracellular A by the calculated amount of intracellular fraction. This is similar to the calculation of the concentration of chloride in red cells when the hematocrit and the concentration of chloride in whole blood and serum are known."

MICROBIOLOGICAL TECHNIQUES

"Perfect as is the wing of a bird, it never could raise the bird up without resting on air. Facts are the air of a scientist. Without them you never can fly. Without them your 'theories' are vain efforts. But learning, experimenting, observing, try not to stay on the surface of the facts. Do not become the archivists of facts. Try to penetrate to the secret of their occurrence, persistently search for the laws which govern them."

PAVLOV

in *Bequest of Pavlov to the Academic Youth of His Country,* Science 83: 369 (1936).

INTRODUCTION

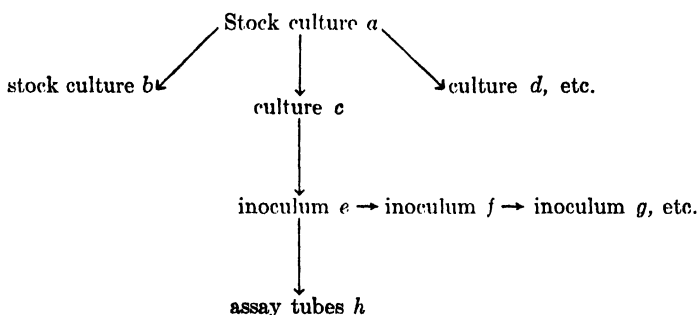
The quantitative assay of a number of biologically important substances, by virtue of their ability to affect the metabolism of certain microorganisms, is still relatively new. The principle involved is the measurement of the influence of the substance on the rate of formation of an end product of the metabolism, such as the carbon dioxide developed by yeast or the acid formed by *Lactobacillus casei.* In other instances the effect on the rate of growth is measured directly by determining the mass or area of a colony, as in the case of the assay of choline with a mutant of *Neurospora crassa.* At the date of this writing, the microbiological methods have been developed mainly for members of the vitamin B family and for amino acids. The great sensitivity of these methods compensates for the fact that in many cases the substances to be assayed occur biologically in very high dilutions. The microbiological technique has been employed almost exclusively on the macro scale. However, no insurmountable problems are associated with the simple reduction in volume and the use of the micro techniques already available, which would be necessary for the adaptation of macro methods to histological or cytological studies. The riboflavin method of Lowry and Bessey (1944) marks a beginning in this direction.

RIBOFLAVIN

The method of Snell and Strong (1939) for the microbiological determination of riboflavin in amounts of the order of 100 mμg. was modified by Lowry and Bessey (1944) so that measurements could be performed in the range of 0.5–2.0 mμg. with a probable error of only about 1% for determinations, in triplicate, of pure riboflavin solutions, and of about 3% for rat cornea. The modifications applied were based on the observations that riboflavin is partially destroyed during autoclaving in small tubes at *p*H 7.0, that air is inhibitory to, while carbon dioxide stimulates the growth and acid production of, the *Lactobacillus casei* used for the assay, that buffers in the *p*H range of 4 to 6 stimulate the growth and acid production of the organism, and that cysteine restores the basal medium if its effectiveness deteriorates on standing after the autoclaving.

Lowry and Bessey Method for Riboflavin

SPECIAL REAGENTS AND MATERIALS

Lactobacillus casei Culture and Inoculum. The procedure for transfer of cultures is shown in the following diagram:

Make the stab transfers *b, c, d,* etc. into yeast-water agar, containing 1% glucose and 0.15 *M* potassium acetate, and, after 24 hr. incubation at 37°, store in a refrigerator. Reserve at least one tube, *b,* as a stock culture. When assays are made on successive days, do not grow inoculum from stock cultures *c* and *d* each day, but transfer a drop of inoculum *e* to a similar tube, *f,* which is incubated for use the next day. Do not use inoculum cultures after they are more than 36 hr. old. Return to a stock culture about every 5 days to minimize the possibility of contamination and variation. Each month prepare new stock cultures corresponding to *b, c,* and *d,* from a tube such as *b* of the preceding month. For inoculum, make a stab from a stock culture into a sterile tube of basal medium containing 0.5–1.0 μg. added riboflavin per 10 ml. Incubate for 24 hr. at 37°, centrifuge out the cells, and resuspend in an equal vol. of sterile 0.9% sodium chloride soln., observing aseptic technique throughout. The most consistent results are obtained from a culture made from a work stab not over a week old.

Basal Medium. Consists of the following: photolyzed sodium hydroxide-treated peptone, 0.5%; glucose, 1%; sodium acetate, 0.6%; cystine, 0.01%; inorganic salts; and riboflavin-free yeast supplement equivalent to 0.1% yeast extract.

(*a*) Prepare the photolyzed sodium hydroxide-treated peptone by exposing a mixture of 40 g. of Difco Bacto peptone (*Difco Laboratories, Inc.*) in 250 ml. water and 20 g. sodium hydroxide in 250 ml. water in a 25 cm. crystallizing dish to light from a 100 watt lamp, with reflector, at a distance of about 30 cm. for 6–10 hr. After standing at room temperature for an additional 14–18 hr. the mixture is neutralized with 27.9 ml. glacial acetic acid, 7 g. anhydrous sodium acetate is added, and the mixture is diluted to 800 ml. Preserve under toluene.

(*b*) Prepare the yeast supplement by adding a soln. of 150 g. basic lead acetate in 500 ml. water to 100 g. Bacto yeast extract (*Difco*) in 500 ml. water; filter off and discard the precipitate, add ammonium hydroxide to a *p*H of about 10, filter off and discard the precipitate, add glacial acetic acid to just acidify the filtrate, precipitate excess lead with hydrogen sulfide, and filter off and discard the lead sulfide. Make up the filtrate to 1000 ml. and store in a refrigerator under toluene. One ml. of the preparation is equivalent to 100 mg. of original yeast extract.

(*c*) Prepare inorganic salt solns. as follows: *Solution A*—Dissolve 25 g. potassium monohydrogen phosphate and 25 g. potassium dihydrogen phosphate in 250 ml. water. *Solution B*—Dissolve 10 g. magnesium sulfate heptahydrate, 0.5 g. sodium chloride, 0.5 g. ferrous sulfate heptahydrate, and 0.5 g. manganese sulfate tetrahydrate in 250 ml. water.

(*d*) Combine 50 ml. of the treated peptone soln., 50 ml. 0.1% cystine hydrochloride, 5.0 ml. yeast supplement, 5.0 g. glucose, 2.5 ml. inorganic salt soln. A, and 2.5 ml. soln. B. Add potassium acetate to give a concentration of 0.3 *M* when the mixture is diluted to 250 ml. With a sterile pipette, add 0.5 ml. of 0.4% freshly boiled neutral cysteine soln. to each 10 ml. of sterile basal medium just before inoculation.

Standard Riboflavin Solutions. Prepare standards containing 0.5, 1.0, 1.5, and 2.0 μg. per 100 ml. in 0.002 *N* hydrochloric acid. Use 0.002 *N* hydrochloric acid for the blank soln. See step 11 under Procedure.

Caprylic Alcohol.

0.1 N Hydrochloric Acid.

0.002 N Hydrochloric Acid.

0.3 N Sodium Hydroxide.

0.030 N Sodium Hydroxide.
0.04% Bromothymol Blue.

PROCEDURE

1. Add 0.1 ml. 0.1 *N* hydrochloric acid, either with a 0.2 ml. graduated pipette drawn out at the end to a slender tip, or with a constriction pipette (p. 172), to the sample containing 2–8 mμg. riboflavin in a 0.75 ml. serological tube (6 × 50 mm.). To clean these tubes boil in half-conc. nitric acid for a few min. and then in distilled water several times.
2. Plug tubes with cotton and autoclave for 15 min. at 15 lb. pressure. Take care to protect from light, particularly while hot. Check weight of several tubes before and after autoclaving to make sure no significant volume change occurred.
3. After cooling, add exactly 0.3 ml. 0.030 *N* sodium hydroxide with a slender-tipped 1 ml. graduated pipette, or a constriction pipette, and mix at once by twirling a fine glass rod with a hooked end in the tube. The soln. now has an excess acid concentration of about 0.002 *M*.
4. Transfer a 0.1 ml. aliquot to each of three serological tubes, and set up, in triplicate, each of the riboflavin standard solns. and the blank using 0.1 ml. of soln. in each tube.
5. Plug tubes with cotton, wrap entire tube rack in black cloth, and autoclave for 15 min. at 15 lb. pressure.
6. When the tubes have cooled, remove black cloth, and with a sterile 1 ml. graduated pipette drawn out at the tip inoculate each tube with 0.1 ml. basal medium which had been previously inoculated with 2 drops of the washed bacterial suspension per 10 ml. Replace cotton plugs and mix by tapping the tubes with the finger.
7. Place rack of tubes in a vacuum desiccator containing damp cotton swabs. Replace the air with carbon dioxide by alternately reducing the pressure to about 150 mm. mercury and adding carbon dioxide back to reach atmospheric pressure. Repeat four to five times and leave pressure at about 700 mm. mercury. Place the desiccator in an incubator for 3 days at 38°.
8. After removing tubes from desiccator, add a minute droplet of caprylic alcohol to each tube to prevent foaming, and blow off the carbon dioxide by carefully bubbling air through the liquid for 1 min. by means of a capillary tube not over 0.5 mm. in diameter.

The bubbles must be small enough so that all spattering is confined in the tube.

9. Add 0.02 ml. 0.04% bromothymol blue and titrate with 0.3 *N* sodium hydroxide from a 0.2 ml. Rehberg burette. Stir by a stream of air bubbles. Neither the air bubbler nor the burette tip should exceed 0.7 mm. diameter. The solubility of the indicator in caprylic alcohol makes it desirable to use very little of the latter.

10. Plot the calibration curve from the titration values found using the standard riboflavin solns. Obtain the riboflavin content of the unknown from its titration value by reference to the calibration curve.

11. To minimize the effect of possible interfering substances, and to obtain a more precise assay, prepare the standards as follows if it is possible: Extract some of the tissue to be analyzed as already described. Irradiate the extract, which is in 0.002 *N* hydrochloric acid, for 30 min. in a Pyrex tube at a distance of 3–4 cm. from a mercury arc lamp, such as the General Electric HB-4, to destroy the riboflavin present. With a minimum of dilution, prepare standards from this extract containing 0, 0.5, 1.0, 1.5, and 2.0 mμg. riboflavin per 0.1 ml.

MECHANICAL SEPARATION OF CELLULAR COMPONENTS

"When one sets out upon the serious and difficult business of wresting from the universe more knowledge of its character and qualities, his efforts must all meet with failure unless he can move with perfect freedom toward the truth wherever the path may lead. No authority must stay him there; no tradition perplex him; no dogma, no prejudice, no vested interest prevent the thorough exploration of every promising avenue he sees. From its beginning, science has struggled to be free from all such man-made bondage."

EDMUND W. SINNOTT
in *Science and the Education of Free Men,* American Scientist 32: 210 (1944).

INTRODUCTION

Centrifugation techniques have become a cytological tool by means of which various cellular components have been separated, and the isolated constituents have been subjected to chemical study in certain instances. The value of this procedure is twofold. The point of view stressed by Bensley (1942) that "to separate separable things before proceeding to their analysis" requires no elaboration or defense. In addition, sufficient material can be isolated in many cases to permit chemical investigations on a relatively macro scale, thus obviating the need for special, and often less available, techniques. Cytochemical work on the separated formed bodies is really just beginning, and a large proportion of the work still must be directed toward perfecting the separations themselves.

As Danielli (1946a) has warned, the centrifugal segregation of particulates from cells could possibly alter the enzyme activities associated with the particulates *in situ*. This may or may not be a factor in specific instances, but in the interpretation of data it should be kept in mind.

The centrifuge microscope, cleverly designed by E. N. Harvey and A. L. Loomis, has enabled direct observation of cells at high magnifications while they are being spun in a centrifuge. In this way it has been possible to determine the manner in which some cellular components become segregated under centrifugal force and to observe the actual scission of certain cells. The instrument and its application, not only to separations, but also to the determination of particular physical characteristics, such as surface forces, have been described by Harvey (1932, 1933).

I. TYPES OF HIGH-SPEED CENTRIFUGES

While the ordinary centrifuge and the Sharples instrument suffice for many separations of cellular constituents, the ultra- or high-speed centrifuge offers particular advantages in other instances. It would carry the present discussion too far afield to give many of the details of the various types of high-speed centrifuges that have been employed for cytological work, but the instruments will be mentioned and the most significant references will be included.

The elaborate Svedberg oil-driven ultracentrifuge, adapted for measurements of physical characteristics of large molecules and various particles, has been the subject of a book by Svedberg and Pedersen (1940) that gives complete details. It has been sufficient for many cytological separations to employ much simpler apparatus such as the gas-driven and electrically powered instruments.

The construction of a centrifuge driven by a high-speed electric motor than can yield a centrifugal force of about 34,800 times gravity at 18,000 R.P.M. was described by Pickels (1942).

A multispeed attachment may be used with the type SB, size 1, centrifuge of *International Equipment Co.* This attachment with head No. 295 may be used for centrifugal forces up to 18,000 times gravity. The full capacity of this head is 84 ml. and the celluloid tubes used with it each has a capacity of 14 ml. (may be obtained from *Lusteroid Container Co., Inc.*). The centrifuge without the multispeed attachment may be used at 1500 times gravity with the horizontal yoke No. 242, which accommodates bottles of 250 ml. capacity, or at up to about 2400 times gravity with the conical head No. 823, which carries 50 ml. tubes.

Gas-Driven "Spinning Top" Centrifuge. This instrument was developed since 1925 when Henriot and Huguenard first spun a small rotor (11.7 mm.) at a speed of 660,000 R.P.M. by means of an air stream that also served as a low-friction cushion to support the spinning rotor. Many improvements have been made in this centrifuge (Beams, 1938, 1940, 1941; McBain, 1939). The instrument

is essentially as shown in the diagram of Figure 156. Figure 157, taken from a review by Beams (1942), demonstrates the effect of the air pressure on the rotor speed for rotors of various designs. Curves A, B, C and D are for a 1⅛ in. rotor with superstructures of various sizes. The highest speed that Beams had obtained by 1942 was almost 1,500,000 R.P.M. with a centrifuge field of almost 8,000,000 times gravity; this was accomplished with a 9 mm. rotor driven by hydrogen. The substitution of hydrogen for air at the same pressure produces higher speeds. The usefulness of the gas-driven centrifuge is greatest when accurate temperature control is not required, and a large centrifugal field is desirable over a small radial distance. It has proved valuable for studies of sedimentation within a cell.

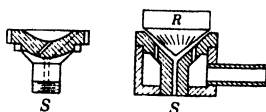



Fig. 156. Diagram of air-driven, air-supported centrifuge. Left: central section through stator cone. Right: section through complete machine. Stator (*S*); rotor (*R*). *From Beams (1942)*

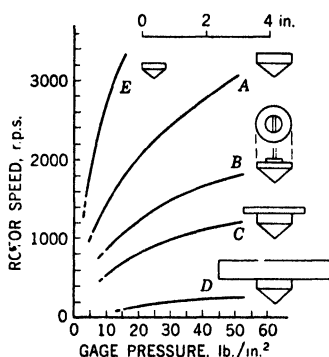



Fig. 157. Relation between rotor speed and driving air pressure for rotors shown at right of curves. *From Beams (1942)*

Plastic-Rotor, Air-Driven Centrifuge. This relatively simple instrument (Fig. 158), developed by Stern (1942), consists of an air driven rotor (*A*) made of a disc of Lucite 0.5 in. thick and 6 in. diameter that has a center axle (*D*) made from $^3/_{16}$ in. drill rod which is supported by Torrington needle bearings (*E*).

A top speed of 17,400 R.P.M., at 48 lb./in.^{2} air pressure, giving a force of 20,200 times gravity has been obtained. The transparency of the analytical fluid cell has enabled direct observation during the centrifugation, when a stroboscopic light source and a low-power

microscope are employed. The instrument is to be made commercially available by *Fisher Scientific Co.*

Vacuum-Type Air-Driven Centrifuge. In this instrument the rotor spins in a vacuum on a flexible vertical shaft. Originally designed by Beams and Pickels (1935), it has since been improved in various ways as described by Beams (1941, 1942). The advantage

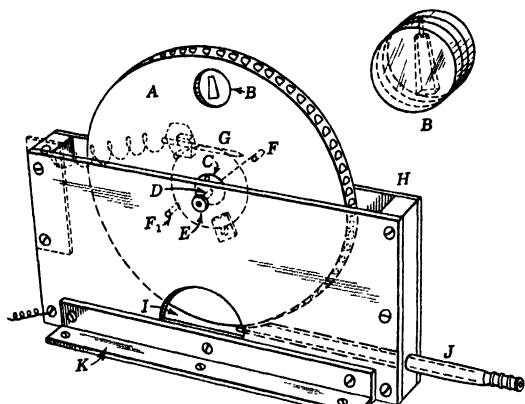



Fig. 158. Air turbine ultracentrifuge with plastic rotor.
From Stern (1942)

Also shown in the figure are: *B*, analytical fluid cell inserted in cylindrical cell hole; *C*, brass disc connected with similar disc on other side of rotor by brass bushing and screws; *D*, fastened to *C* and turned down and surface-hardened at ends to fit *E* mounted in casing, *H*, and carefully aligned with bearing on opposite side; *F*, F_1, brass contacts, inserted in rotor surface; *G*, contact brush, made from spring bronze, insulated from casing *H*, adjustable in position; *H*, centrifuge casing, made from sheet brass; *I*, semicircular opening in casing *H* to permit free escape of expanded driving air; *J*, air jet, $^{7}/_{32}$ in. lumen, trumpet-shaped at inlet end and conforming with rotor shape at outlet end; *K*, angle for mounting on wooden base. Insert *B*, analytical fluid cell, made by cementing, with Lucite cement, two outer discs of colorless Plexiglas resin to central disc of red Plexiglas into which a sector-shaped opening of 12 mm. height and 3 mm. depth has been cut, connected with periphery by narrow drill hole, through which the solution under study is introduced with a hypodermic syringe. When in use the cell is inserted into cell hole in rotor center and the broad base of the sector pointing toward the periphery. During operation, the centrifuge is covered by a steel guard, made from 0.5 in. thick boiler plate by welding, equipped with openings opposite the cell holes and slots near the base to permit escape of air stream.

of this construction over the nonvacuum type is that it obviates the undesirable effects of air friction and the need for great precision in the dynamical balance of the rotor. Furthermore, it has the capacity for handling much greater volumes of material. On the other hand, it is considerably more complicated mechanically.

Electrically Powered, Magnetically Supported Vacuum-Type Centrifuge. In order to overcome the irregularities occasioned by variations in gas pressure in the preceding types of centrifuge, various methods have been employed to regulate the pressure, but the most successful among these have been rather complicated. Hence Skarstrom and Beams (1940) devised an electric centrifuge in which the rotor was supported magnetically and allowed to spin in a vacuum. For constancy of speed and ease of operation this instrument appears to be superior to gas-driven vacuum types. Refinements of the apparatus have been appearing continuously from Beams' laboratory at the University of Virginia. In more recent models the centrifuge is suspended from a vertical iron rod supported by the field of a solenoid. An automatic regulatory mechanism is employed whereby the current in the solenoid is controlled by the height of the rotor, decreasing as the rotor rises and increasing as it falls. In this fashion the height of the rotor is kept constant and free from any mechanical contact. Consequently, when the rotor is spun in a vacuum by a rotating magnetic field, extremely high speeds can be obtained. With this technique MacHattie (1941) spun a $^{3}/_{32}$ in. steel ball at 6,600,000 R.P.M., and when the driving field was cut off at 6,000,000 R.P.M., it lost only about 1% of its speed in an hour.

II. SEPARATION OF COMPONENTS OF A. PUNCTULATA EGGS (AFTER HARVEY)

The eggs of the sea urchin, *Arbacia punctulata,* have been employed particularly for cytophysical and cytochemical investigations. Therefore, it may be well to include here a short description of the procedure developed by Harvey (1932, 1936) for the separation of components of this cell, especially since similar treatment has been applied to the ova of other marine invertebrates and to other cells employed for laboratory study.

Centrifugation is carried out in tubes, a little larger than hematocrit tubes, having a capacity of 0.7 ml. Place the eggs in 1 vol. sea water over a layer of 2 vol. 0.95 *M* sucrose soln. (95% of 342 g. sucrose added to 1 l. tap water) in the centrifuge tube. Roll the tube gently to effect partial mixing. Centrifuge the eggs in this medium, which is isosmotic and isopycnotic with the eggs, for 3–4 min. with a centrifugal force of 10,000 times gravity. Three layers form with the white halves above, under them a pinkish layer of elongated whole eggs, and on the bottom the red halves. With more sucrose solution, further centrifugation results in breaking of the halves into quarters, as indicated in the diagram in Figure 159.

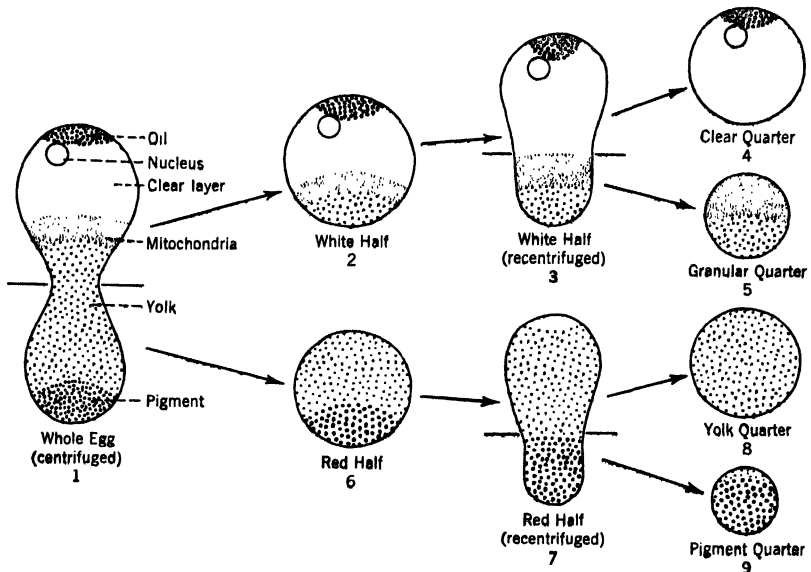



Fig. 159. Unfertilized egg of *Arbacia punctulata,* stratified by centrifugal force (about 3 min. at 10,000 g.), and the halves and quarters into which it breaks. Drawn from camera lucida sketches and photographs, as accurately as possible to scale (magnified ×275). The clear area in 7 at the centripetal pole is due to further packing of the granules with longer centrifuging. *From Harvey (1936)*

III. ISOLATION OF CELL NUCLEI

The isolation of cell nuclei for purposes of chemical investigation seems to have been attempted first by Miescher (1871), who digested leucocytes with pepsin to remove the cytoplasm; the nuclei could then be collected on a filter. Other early attempts at the separation of nuclei were made by Ackermann (1904), who laked chicken erythrocytes in distilled water and precipitated the nuclei in 3.6% sodium chloride solution, and Warburg (1910), who employed a freezing-thawing technique to disrupt the red cells.

Since a number of methods for the isolation of nuclei have been evolved during the past 15 years, each with its particular advantages and drawbacks, and each more suited to certain types of cells than to others, the most important of these methods will be described in detail so that the investigator may use his own judgment in their application.

Since the Warburg (1910) method of freezing and thawing results in partial agglutination and damage to erythrocytes, and incompletely hemolyzes them, Laskowski (1942) developed a technique based on hemolysis by lysolecithin. In some instances, as in the case of lipid studies, lysolecithin treatment would be undesirable; hence Dounce and Lan (1943) employed saponin for the hemolysis.

A. NUCLEI FROM AVIAN ERYTHROCYTES

Laskowski Procedure for Isolation of Nuclei

Centrifuge the erythrocytes from 30–40 ml. citrated blood, and wash them several times with isotonic saline soln. by successive centrifugations and decantations. After the last centrifugation, pipette out the red cells from the bottom of the layer and suspend them in 30–40 ml. saline soln. Add 5–8 ml. lysolecithin soln. and determine when hemolysis is complete by microscopic examination; it usually takes 20–40 min. at room temperature. Centrifuge the liquid, and wash the material thrown down five to six times with

saline soln. by resuspending in 30–40 ml. and centrifuging each time. The final suspension of nuclei has a faint yellow color, and it is stable for 2 weeks when stored in a refrigerator. The nuclei may be stained with aqueous methylene blue. Agglutination occurs when the suspension is diluted with water but the nuclei may be suspended safely in 0.1 *M* potassium dihydrogen phosphate soln.

The lysolecithin is prepared by grinding the poison glands of 100 bees with an emulsion of 5 g. of lecithin in 20 ml. phosphate buffer (*p*H 7.0–7.1) and allowing the mixture to digest at 37° for 24 hr. The material is then filtered through a Berkfeld filter. Snake venom could be substituted for the bee poison.

NOTE. In a private communication to the writer, R. R. Bensley pointed out that "it seems a pity to spend the time to pull the stings out of a hundred honey bees when a few drops of ether added to a suspension of the cells in salt solution will accomplish the same purpose. As a matter of fact, this process of hemolysis does not isolate the nuclei since the stroma of the corpuscle can be demonstrated closely contracted around the surface of the nucleus."

Dounce and Lan Procedure for Isolation of Nuclei

Wash the red cells from fresh defibrinated blood twice with 0.9% sodium chloride soln. by centrifuging and decanting. Suspend in a vol. of the saline soln. equal to that of the blood used and add 5 ml. of 0.11 *M* phosphate buffer (*p*H 6.8–7.0), containing 0.3 g. Merck's purified saponin for each 100 ml. red cell suspension. The laking is complete in 5 min. Wash the nuclei liberated four to five times by centrifuging, decanting and resuspending in saline soln. Each time add 2–3 ml. of the 0.11 *M* phosphate buffer to the centrifuged nuclei, without stirring, just before adding the saline soln.

The observation of Crossmon (1937), that nuclei are ejected from muscle cells when a bit of the tissue is teased in 5% citric acid, led Stoneburg (1939) to an application of this principle for the isolation of nuclei from beef heart muscle, rabbit thigh muscle, tumor cells, and leucocytes. Dounce (1943a) has pointed out that while the use of 5% citric acid may yield nuclei satisfactory for studies on lipids, nucleic acids, and acid-resistant nucleoproteins, the low *p*H involved denatures enzymes and proteins in general. Hence, Dounce (1943a) modified the Stoneburg procedure to obtain nuclei suitable for enzyme and protein work.

B. NUCLEI FROM OTHER CELLS

Stoneburg Procedure for Isolation of Nuclei

Nuclei from Muscle Tissue. Grind the tissue, cleaned of visible fat, as fine as possible in a meat chopper. Place 100 g. in a 1 l. beaker and cover with 500 ml. of 5% citric acid. Stir occasionally and let stand overnight. Skim off surface fat, dilute soln. with an equal vol. water, and filter through eight layers of cheesecloth. Run filtrate through a Sharples centrifuge at 26,000 R.P.M. The centrifuge cylinder is lined with cellophane to prevent the solvent action of the acid on the metal. The material washed off the cellophane sheet will contain about 50% nuclei. Wash the material four times by successive centrifugations and resuspensions in water. The nuclei content will be raised to about 70% by this process. Digest the residue in the centrifuge bottle with 250 ml. 1% hydrochloric acid and 250 ml. 0.8% sodium chloride soln. containing 2–4 g. pepsin (strength 1:10,000) for 4 hr. at 37°. Stir occasionally, and at the end siphon off the supernatant from the settled nuclei. Wash the nuclei with distilled water and then centrifuge. A smear of the residue should show nuclei undamaged histologically and free from debris.

Nuclei from Rat Tumor Tissue. After removal of necrotic material the tissue is ground in a meat chopper, treated with citric acid soln., and filtered as in the case of muscle. Add 1 vol. water to the filtrate and let stand 3 hr. Omit supercentrifugation but otherwise continue the procedure given for muscle.

Nuclei from Leucocytes. Add pus to five times its vol. of 5% citric acid. The nuclei settle rapidly and are then subjected to the procedure given for tumor tissue.

Dounce Procedure for the Isolation of Nuclei

Nuclei from Rat Liver. Make up approximately equal vol. cracked ice and water to 500 ml. and add 1.05 ml. 1 *M* citric acid (final conc. *M*/475, *p*H 6.0–6.2). Place the mixture in a Waring blendor and add 100 g. frozen rat liver as rapidly as possible without stalling the stirrer. The liver may be frozen conveniently in the freez-

ing compartment of a refrigerator and it should be used as soon as frozen. Run the blendor until all the ice has melted (10–15 min.). Strain the mixture through two layers of fine cheesecloth (twenty threads per cm.) and, when the cloth becomes clogged, replace it with new material. Wring out the cloths used and strain the liquid into the already strained batch. Repeat the straining with four layers of cheesecloth and centrifuge for 20 min. at 1500–2000 R.P.M. in 250 ml. centrifuge bottles. Decant down to the first line demarking the supernatant and the more loosely packed sediment. Add distilled water to the sediment to make a final vol. of 400 ml. and stir well to break up lumps. A drop of caprylic alcohol may be used to break the foam. Centrifuge the suspension for 15 min. and again discard the supernatant fluid. Stir the residue with about 400 ml. distilled water and centrifuge for 10 min. Wash sediment with 200 ml. distilled water and centrifuge 5 min. at a slower speed (1000–1500 R.P.M.). Discard the supernatant and stir the nuclei with 200 ml. distilled water. Centrifuge for only 3 min. this time and at a still slower speed. Repeat the washing twice with 200 ml. portions of distilled water and centrifuge 3 min. each time at the lower speed. Before the last centrifugation, pass the suspension through four layers of cheesecloth. Stir the nuclei well with about 100 ml. distilled water and let stand for 45 min. in a 100 ml. cylinder. Carefully decant the top 95 ml. containing most of the nuclei from the whole cells on the bottom. Recover the nuclei by centrifuging and resuspending in a small vol. distilled water. The light reddish-brown color of the final preparation is due to adsorbed hemoglobin.

There are some additional points that should be mentioned. At a *p*H 6.5, or higher, the cells rupture in the presence of distilled water but the purified nuclei do not seem to be harmed. In the *p*H range of 4.0 to 5.9 cytoplasmic granules agglutinate to a solid mass on centrifugation making it impossible to separate nuclei, while at 3.8 to 4.0 good nuclei preparations are obtained but the acidity results in some alteration of enzymes and proteins. Adsorbed hemoglobin can be removed from the product to some extent by one washing, and completely by two, with Ringer soln. at *p*H 7.4. However, the Ringer soln. extracts a certain amount of protein from the nuclei and causes them to shrink. Subsequently, Dounce (1943b) showed that in the preparation of nuclei at *p*H 3.8 to 4.0 the *p*H tends to rise above 4.0 on successive washings with the result that the nuclei agglutinate.

This may be prevented by adding a few drops of 1 *M* citric acid to the wash water each time. Actually less washing is required at *p*H 3.8 to 4.0 than at 6.0 to 6.2 since the nuclei pack better on centrifuging.

Nuclei from Tumor Tissue. In a later paper Dounce (1943c) described the application of the preceding method to tumor cells. In the case of Walker carcinosarcoma 256, add 37.6 ml. 1 *M* citric acid in the blendor to the 500 ml. of mixture containing 100 g. tissue. Run the blendor for 15 min.; the final *p*H is 3.0. After the first washing, add a drop or more of 1 *M* citric acid to prevent agglutination. The reason for the lower *p*H in this instance is that cytoplasm cannot be removed very well from the nuclei at *p*H 3.8 to 4.0. When hepatoma 31 was used, it was impossible to obtain a good preparation in the *p*H range 3.0 to 4.0 necessitating the addition or 100 ml. 1 *M* citric acid to the 500 ml. vol. The final *p*H was 2.4. Here, too, citric acid is added in the washings to prevent agglutination.

The Dounce procedure has been criticized by Hoerr (1943), who claims that 0.85% sodium chloride is preferable to distilled water for extractions and washings, and who objects to the use of the Waring blendor as being too drastic a treatment leading to a certain degree of gelling. While Hoerr may be right, and even though it is obvious that the less drastic the treatment of biological systems to attain a given end, the better, the fact that Dounce obtained good preparations which were suitable for a variety of studies on enzymes and other nuclear constituents cannot be disregarded. Hoerr favors the Lazarow (1941, 1943) procedure of breaking up cells by forcing a suspension of them through bolting silk, and handling the material at a temperature just above 0° taking care not to let it freeze.

Lazarow Procedure for Isolation of Liver Nuclei as Used by Hoerr

Perfuse the liver *in situ* with cold physiological salt soln. by repeated variations of hydrostatic pressure from 2 to 4 ft., clamping the inferior vena cava for 10–20 sec. each min., and massaging the liver through the abdominal wall while the blood is flowing freely from the vein. Usually 500–700 ml. saline soln. is required to remove practically all the blood cells. Remove the liver and chill to 0° as rapidly as possible, but do not allow to freeze. Triturate the tissue briefly in a mortar with 0.85% sodium chloride soln. and gently knead through bolting silk. Suspend the emulsion in 0.5 to 0.7% sodium chloride soln. at a *p*H of 6.0 to 6.2. Separate the nuclei from por-

tions of this medium by successive centrifugations at 1500 R.P.M., carrying out all the operations in a cold room at 0°. Follow the degree of purification by making wet smears of both supernatant and sediment after each centrifugation. Smears may be stained after fixing in osmic acid vapor.

The principle of separating cellular components by centrifugation in nonaqueous media (benzene-carbon tetrachloride mixtures) of controlled specific gravity was first applied by Behrens (1932) to the isolation of nuclei from calf heart, muscle, and thymus (Behrens, 1938). Fuelgen, Behrens, and Mahdihassan (1937) used this technique to obtain nuclei from rye germ cells, and later Behrens (1939) employed the same procedure for the separation of nuclei from liver cells. In this country Mayer and Gulick (1942) used a modified Behrens technique to isolate nuclei from bovine thymus cells, and Williamson and Gulick (1944) applied the method to other cells having a large proportion of nucleus such as those of human tonsil and bovine supermammary lymph gland. The limitation should be borne in mind that the benzene-carbon tetrachloride mixtures will extract lipids from the nuclei. It is claimed that proteins are not significantly affected. In general the technique seems to involve a great deal of time and manipulation, and it is too drastic a process for many purposes.

Behrens Procedure for Isolation of Nuclei from Thymus and Lymph Cells (as Modified by Gulick *et al.*)

Cut the tissue into pea-size bits and freeze in liquid air as soon as possible after removing from the animal. Dehydrate by treatment with a number of changes of 10 vol. portions of dry acetone cooled below —20°. Remove remaining fat by continuous extraction with ether that has been dried over anhydrous sodium sulfate; after the extraction, remove the residual ether in a vacuum desiccator over conc. sulfuric acid. Grind the dry material in a power mill until it can pass a 40 mesh sieve. After suspending the powder in a benzene-carbon tetrachloride mixture of specific gravity 1.25, comminute further in a ball mill with glass beads. It usually takes 4–8 weeks at 30–50 R.P.M. to effect separation of the nuclear and cytoplasmic particles. Test for the completeness of this separation from time to time by staining a drop of the suspension with hematoxylin and eosin in the following manner:

Dry a smear of the suspension on a glass slide, and place for 1 min. in a filtered fresh mixture of equal vol. of 1% yellowish eosin and Harris ripened alum hematoxylin. Transfer to citric acid-sodium

phosphate buffer (*p*H 3.9) and, after 3 min., dry, clear with immersion oil, and examine microscopically. Eosin stains the cytoplasmic particles, and hematoxylin the nuclear material.

Before separating the suspended cellular components, remove connective tissue by several sedimentations in pure benzene. Discard the benzene carrying the connective tissue fragments each time. Then centrifuge the cellular powder from progressively denser mixtures of benzene and carbon tetrachloride, retaining the sediment in the tube each time. When the proportion of carbon tetrachloride has been increased to the point where no more powder settles, add benzene until the denser nuclear fraction begins to come down under centrifugation. At this final separation, the specific gravity of the suspension medium was between 1.345 and 1.350 at 28° when bovine thymus cells were used. Nuclear concentrates prepared in this manner were found to possess less than 5% contamination. The product is a fine granular light tan dust that is quite hydroscopic, and it is obtained in 4.3 to 6.7% yield on the basis of the dry weight of the original tissue. Highest yields are given by thymus material.

IV. ISOLATION OF CHROMATIN THREADS FROM CELL NUCLEI

Claude and Potter (1943) succeeded in isolating chromatin threads from the nuclei of spleen cells from leukemic mice and of liver cells from normal guinea pigs and rats. Apparently these threads are related to the chromosomes. On standing, especially in very dilute salt solution, the filaments disintegrate and seem to be replaced by free refractile granules. In distilled water the threads swell rapidly and finally disappear.

Claude and Potter Procedure

Care should be taken to conduct all the operations in a cold room at 0–5°. Grind gently batches of 20–30 g. of the cold tissue with an equal weight of sand in a mortar for 3 min., and progressively add six times the tissue weight of either distilled water or 0.9% sodium chloride soln. having a *p*H of 7.4. Centrifuge the suspension for 1 min. at 1500 times gravity to throw down the sand and tissue debris. Centrifuge the supernatant fluid for 10 min. at 1500 times gravity to sediment the thread-like material, and discard the supernatant. Suspend the material from 25 g. of tissue in 35 ml. of the saline soln. by gentle shaking, and centrifuge by bringing to the speed corresponding to a force of 1500 times gravity and then cutting off the current in the centrifuge motor. This short "up-and-down run" throws down any remaining sand and tissue debris. Subject the supernatant fluid to another 10 min. centrifugation at 1500 times gravity and follow by resuspension of the sediment in 35 ml. saline soln., another "up-and-down run," and a final 10 min. centrifugation of the supernatant fluid to bring down the white mass of chromatin threads.

V. ISOLATION OF CYTOPLASMIC PARTICULATES

A. MITOCHONDRIA

The mitochondria, dispersed throughout the cytoplasm of cells, are microscopically visible and have been known for some time. However, their isolation was not accomplished until 1934 when Bensley and Hoerr succeeded in separating them from liver cells of the guinea pig by differential centrifugation. The substantial gap in time between the recognition of mitochondria as structural entities in the cytoplasm, and their isolation, was largely due to their highly labile character, at least in cells of some animals. Mitochondria rapidly disappear from the cells after death, and they also disappear when tissue is treated with organic solvents or fluids containing appreciable acetic acid, and when the temperature is elevated to 48–50°. An exception has been found, in the case of mitochondria of the liver cell of *Amblystoma,* by Bensley and Gersh (1933b), who observed that, after freezing-drying the tissue, extraction with organic solvents, treatment with acetic acid, or elevation of temperature did not cause the disappearance of these bodies. The particles called "secretory granules" by Claude (1943a) include mitochondria. Subsequently Claude (1946) referred to particles from liver cells of 0.5–2.0 μ diameter as "large granules" which consist "of secretory granules and mitochondria." Some additional details for the isolation procedure of Bensley and Hoerr (1934) have been given by Hoerr (1943) and are incorporated in the following description. Claude (1944) and Claude and Fullam (1945) employed certain neoplastic cells of the rat as a source of mitochondria, and Claude (1946) gave a detailed description of the separation of particulates from liver.*

Bensley and Hoerr Procedure for Guinea Pig Liver

The entire procedure should be carried out in a cold room at 0°. Remove blood from the liver by perfusion with cold 0.85% sodium

* Since this writing, Hogeboom *et al.* published a paper on the isolation of mitochondria from rat liver; see Bibliography Appendix, Ref. 55.

chloride soln. of *p*H 6.0 to 6.2. Grind the tissue gently in a mortar and knead through bolting silk. Suspend the liver emulsion (about 30 g.) from the liver of a 400–500 g. guinea pig in no more than 200 ml. of the physiological saline soln. and centrifuge for 30 min. at not over 600 R.P.M. Centrifuge the supernatant fluid once or twice at 1500–2000 R.P.M. for not over 1 min. Centrifuge the supernatant for 10 min. at 2000 R.P.M. and suspend the sediment in 200–300 ml. of the saline soln. Repeat this washing process until the supernatant is free of soluble protein; it usually requires four or five washings.

Follow the degree of isolation at various steps by fixing a smear of the material on a slide with osmic acid and staining with aniline-acid fuchsin and methyl green. The distinct yellowish color of the unstained mitochondria can also be employed as something of a check on the separations.

The lower tip of the cake in the centrifuge tube may be contaminated with cell fragments; if so, cut off and discard this portion after drying. Finally wash the mitochondria with distilled water containing 1 drop 1 *N* acetic acid in 200 ml.; centrifuge, and dry the sediment in the centrifuge tubes *in vacuo* over phosphorus pentoxide.

During the preceding treatments the mitochondria undergo some swelling and change from rods to round granules. The granules do not coalesce and clump unless the centrifugation is at too high a speed, which results in fragmentation and consequent agglutination.

Claude Procedure for Certain Neoplastic Cells of the Rat

The cells used as the source of mitochondria were obtained from 10–15 g. tumors that develop within 10–12 days after subcutaneous inoculation of leukemic cells into rats. The advantage of this source of material is the uniformity of the cell type, the relative lack of connective tissue, the scant blood supply, and the absence of appreciable necrosis. Furthermore, the mitochondria compose the major portion of the large cytoplasmic granules.

The following process should be carried out in the cold (2–8°). Chill freshly removed tumors, pass through a 1 mm. mesh masher, and grind the pulp for 3–5 min. in a mortar. Add, very slowly at first, a 0.85% sodium chloride soln., buffered to *p*H 7.2 with phosphate having a final concentration of 0.005 *M*, to a total volume equivalent to five times the weight of tissue pulp. (It is important to main-

tain the slightly alkaline reaction to prevent clumping of cytoplasmic components.) Centrifuge the cellular suspension at 1500 times gravity for 4 min., and follow by a 10 min. run, in order to throw down most of the debris, unbroken cells, and nuclei. Separate the mitochondria from the supernatant fluid by centrifuging at 2400 times gravity for 25 min., or else at 18,000 times gravity for 4 min. Discard the supernatant liquid and resuspend all but the bottom portion of the sediment in buffered saline soln. Wash the mitochondria by two to three sedimentations and resuspensions in buffered saline soln. discarding the bottom layer after each centrifugation to eliminate erythrocytes or nuclei that may have escaped earlier separation. The final mitochondria suspension consists of granules 0.5–1.5 μ in diameter. The centrifugations were conducted in the type SB, size 1, centrifuge of the *International Equipment Co.* (page 448).

Claude Procedure for Isolation of "Large Granules" from Liver

Rat livers are used which have been depleted of blood by bleeding, as well as guinea pig livers which have been perfused from the portal vein or aorta with physiological saline solution. In the latter instance the animals are prepared by placing them under ether anesthesia and injecting heparin intravenously, or directly into the heart, in a dose of 1–2 mg. per 100 g. body weight. All the operations in the preparation of the extract and the separation of the "large granules" are carried out in a cold room at around 0°, and all the solutions are employed at this temperature.

Preparation of Extract. Chill livers immediately after removal and force through a tissue masher fitted with a 1 mm. mesh screen. Grind 60–80 g. of the pulp in a mortar about 5 min. and add dropwise 0.85% sodium chloride (made slightly alkaline to prevent agglutination by adding 0.2 ml. 1 *N* sodium hydroxide/l.) until 20–30 ml. have been introduced. Then add the solution more rapidly until a final volume is obtained equivalent to five times the weight of the liver pulp used. Centrifuge the suspension at 1500 times gravity for 3 min. and discard all the sediment. Centrifuge the supernatant twice more for 3 min. at 1500 times gravity and discard the deposit each time. The resulting supernatant liver extract is used for subsequent separations.

Separation of "Large Granules." Centrifuge the liver extract for 25 min. at 2000 times gravity. Resuspend the sediment, except

for the portion at the very bottom which consists of nuclei, red cells, and debris, in a small amount of the "supernate" which has been left in the tube after decanting the bulk of it. Centrifuge this suspension for 30 min. at 2000 times gravity and withdraw the "supernate" by suction with a capillary pipette. Take up the sediment in enough alkaline saline solution to bring the total volume to one-twelfth that of the liver extract; hence the volume at this point is usually 20–25 ml. Leave the small disc of packed debris and nuclei in the tube and do not suspend it with the rest of the sediment. Finally dilute 15–20 ml. portions of the suspension which contains the "large granules" to 35–45 ml. with alkaline saline and centrifuge for 30 min. at 2000 times gravity. The sediment of "large granules" may be resuspended in alkaline saline and recentrifuged in the same manner for an additional washing, and the washing process may be repeated several times. The "large granule" fraction represents about 10–15% by dry weight of the total solids in the liver extract. Suspensions of "large granules" become increasingly acid on standing and may require addition of alkali to maintain neutrality.

Instead of the use of 2000 times gravity to effect separation of "large granules" from the extract a force of 18,000 times gravity for 3–5 min. periods will give better fractionation. Celluloid tubes of 14 ml. capacity were used in the high-speed attachment of the International centrifuge (page 448) for this purpose.

B. SUBMICROSCOPIC PARTICULATES

Claude (1940) discovered and separated submicroscopic particulates from saline extracts of embryonic chick tissue, and subsequently separated these bodies from a wide variety of other tissues of various species as well, Claude (1943b). Originally, Claude considered that these cellular units might be mitochondria or their fragments, but has since agreed that this is unlikely because the particles are much too small. Claude (1943a) proposed that the term "microsome" be applied to this cellular entity; it has also been referred to by others as the submicroscopic lipoprotein complex. A second submicroscopic particulate, composed of glycogen, was demonstrated by Lazarow (1942, 1943) in the guinea pig liver cell.

Lazarow Procedure for Separation and Isolation of Lipoprotein and Glycogen Particles from Guinea Pig Liver

Prepare a suspension of fragmented liver cells in the manner described for the preparation of mitochondria using a volume of saline three times that of the liver (page 462). Continue all operations in a cold room. Clarify the suspension by spinning for two 10 min. periods at 3000 R.P.M. in an 8 in. angle centrifuge, followed by 15 min. at 6000 R.P.M. Discard the precipitates after each centrifuging. Transfer the final supernatant fluid to clean 10 ml. Lusteroid test tubes and centrifuge at 12,000 R.P.M. for 30 min. The sediment consists of a densely packed cake of the particulate glycogen, and a loosely packed red precipitate which can easily be separated from the glycogen by inverting the tube. The red precipitate is a mixture of the glycogen and lipoprotein particles. After removing the red material, wash the surface of the packed cake twice with physiological saline soln. and then resuspend in saline allowing 30 min. for dispersion. Centrifuge at 12,000 R.P.M. for 30 min. and discard the supernatant. Repeat the resuspension and centrifugation four times. Dry the final cake in a vacuum desiccator and a clean white powder consisting mainly of glycogen is obtained.

The red precipitate containing both lipoprotein and glycogen can be freed of the latter by digestion with a purified diastase preparation. The separated fractions of both submicroscopic particulates form transparent gelatinous pellets on centrifugation.

Claude Procedure for Isolation of "Microsomes"

All operations are carried out in a cold room at 0° and all solutions employed are cooled to this temperature.

Suspend the ground tissue mass in eight to ten times its weight of either 0.005 *M* phosphate buffer, *p*H 7.1, or 0.0002 *N* sodium hydroxide and centrifuge 20 min. at 2400 times gravity. Subject the supernatant fluid to high-speed centrifugation at about 18,000 times gravity for 1 hr. Take up the sediment in a little water and centrifuge at the top speed for 3–5 min. to throw down the coarser particles and then resuspend them and again centrifuge for 3–5 min. Combine the supernatants from both short runs and repeat the process of a 1 hr. run followed by two short runs two or three times. In this fashion a concentration of particles ranging in diameter from

60 to 200 mμ, which will include the "microsomes" will be obtained.

In the case of liver tissue, the following procedure has been followed (Claude, 1946): To the "supernate" obtained when the "large granules" are separated from the liver extract (page 465), add 0.1 *N* sodium hydroxide to bring the *p*H to 7.2 to 7.4. Centrifuge for 4 min. at 18,000 times gravity and discard the sediment which contains "large granules" not previously separated. Bring the "supernate" to *p*H 7.2 to 7.4 if necessary and spin down the "microsomes" by centrifugation for 1.5 hr. at 18,000 times gravity. Wash the "microsome" fraction which appears as a jellly-like pellet by resuspending in neutral saline soln. and centrifuging for 1.5 hr. at 18,000 times gravity. After a second washing the "microsome" yield on a total solids basis is 10–20% of the original liver extract.

VI. ISOLATION OF CHLOROPLASTS FROM LEAF CELLS

Chloroplastic material was separated from spinach leaves by Chibnall (1924), who ground the leaves in water, removed liquid by squeezing through a silk bag, and filtered the fluid through paper pulp. The chloroplastic material mixed with other cellular constituents was retained on the filter. Menke (1937) found that ammonium sulfate would precipitate chloroplastic material from an acidified suspension prepared by grinding spinach leaves in water. The most satisfactory preparations are obtained by differential centrifugation. This procedure was adopted by Menke (1938), who employed *M*/15 phosphate as the suspension medium, Mommaerts (1938), who used water containing a little calcium carbonate, Granick (1938), who found 0.5 *M* glucose solution particularly suitable, and Neish (1939), who also used 0.5 *M* glucose to obtain intact chloroplasts and distilled water if merely chloroplastic material was desired. Comar (1942), found that freezing decreases the solubility of chloroplastic substance, and he demonstrated that, in a suspension that had been previously frozen, only a few minutes' centrifugation at 3700 R.P.M. was required to bring down the substance.

The importance, in some cases, of the time of day the leaves are picked was emphasized by Hill and Scarisbrick (1940), who found that if the leaves of *Stellaria media* were picked at 10:00 A.M. active chloroplasts could be isolated, but if picked later in the day the activity fell, approaching zero on sunny afternoons.

The procedures employed by Granick (1938) and Neish (1939) will be described. Galston (1943) showed that Granick's method was applicable to fibrous grass leaves such as those of the oat plant. Neish successfully employed his method with leaves of *Trifolium pratense,* red clover, *Elodea canadensis,* and *Arctium minus,* common burdock, and *Onoclea sensibilis,* sensitive fern but fibrous or mucilagenous leaves such as those from couch grass or basswood did not yield satisfactory preparations.

Granick Procedure

Weigh rapidly on a torsion balance 3 g. fresh leaf tissue, excluding mid ribs and main veins. Place the tissue between wet paper toweling until ready for use. In this manner the cells absorb water, become turgid, and are more easily torn apart. After removing from the paper, wash the tissue with distilled water, dry superficially, and place some of the material in a 150 ml. porcelain mortar containing 1 g. sand and 25 ml. 0.5 *M* glucose soln., cooled to about 5°. Rub gently until the liquid becomes dark green. Add more tissue and continue the cellular disruption. Pour the suspension into a 50 ml. centrifuge tube. Add 20 ml. cold glucose soln. to the residue in the mortar, and after further grinding add the liquid to that in the centrifuge tube. The time and rate of centrifugation depends on the leaf material employed. The process and the number of centrifugations required must be controlled by microscopic examination of the centrifugates under an oil immersion objective.

Neish Procedure

Remove as much of the fibrous material as possible, wash the leaf tissue with distilled water, squeeze out excess water by hand, and cut about 20 g. of the compressed mass with scissors into an 8 in. porcelain mortar. Mash to a pulp, add about 200 ml. distilled water in three portions, grinding after each addition, and filter the mixture through 200 mesh bolting silk. Centrifuge the filtrate in 250 ml. tubes at 2000 R.P.M. (With material prepared from sensitive fern a lower speed is required since the larger chloroplasts in this species sediment more rapidly.) The starch granules settle faster than the chloroplasts and hence may be separated from them at this point. Decant the supernatant fluid containing the chloroplasts into a 1 l. graduated cylinder until 950 ml. is obtained, then add 2 *M* calcium chloride soln. to the 1 l. mark and mix the whole. After 30 min. the flocculated chloroplasts settle to about the 200 ml. level. Discard the supernatant fluid and centrifuge the flocculated material at the same speed as before. Remove the supernatant fluid and triturate the chloroplasts with a glass rod fitted with a rubber policeman. Repeat the centrifugations and washings until the concentration of the calcium chloride is reduced to the point at which the chloroplasts again begin to disperse in the liquid. The material is collected after a final

centrifugation. To obtain intact chloroplasts, substitute 0.5 *M* glucose soln. for the distilled water in the procedure and centrifuge the chloroplasts out at high speed without using the flocculating agent. (Although nothing is said about it in Neish's paper, the general experience with chloroplast isolation would suggest that the separations be carried out in the cold.)

VII. ISOLATION OF OTHER PARTICULATES FROM CELLS

In addition to those already considered, a wide variety of particulates from different types of cells has been isolated by differential centrifugation. Without going into the details in each case, a number of the particulates will be mentioned.

Sevag, Smollens, and Stern (1941) isolated a green particle from *Streptococcus pyogenes* by first disrupting the organisms by sonic vibrations or grinding in a ball mill, precipitating the particles by 66% saturated ammonium sulfate, and finally subjecting the resuspended particles to repeated low- and high-speed centrifugations. The green particulate is sedimented in a gravitational field of 60,000–90,000 times gravity in 1 hr. The final yield amounted to 0.43%.

The iron protein, ferritin, was isolated from horse liver by Stern and Wyckoff (1938a, 1938b). Several hours' centrifuging at about 70,000 times gravity is required to bring the ferritin down. This particulate is unique in having an extremely high sedimentation constant, and an iron content of about 20%. The average particle size is 10 mμ.

The association of cytochrome oxidase activity with macromolecular particles separated from heart muscle was described in a review by Stern (1943); Stern also included a description of the centrifugal segregation of particles bearing the activity of Rous sarcoma agent, fowl leukemia virus, and dysentery bacteriophage.

Sonic vibrations, produced by the instrument of Chambers and Flosdorf (1936), were successfully employed by Henle *et al.* (1938) and Zittle and O'Dell (1941) for the disruption of spermatozoa into heads, midpieces, and tails, which could then be separated by differential centrifugation. Henle's group found that 7 min. of vibration at 9000 cycles per second of an intensity that promotes vigorous cavitation in the fluid was sufficient to break up the sperm of bull, dog, and rabbit; 15 min. was required for guinea pig sperm and 20 min. for human. The temperature was kept below 20° by water cool-

ing the inside of the nickel vibrating element. Difficulty was experienced with separation of the parts of guinea pig sperm, since the heads and tails come down at almost the same rate on centrifugation giving fractions containing 12–15% of the unwanted portion in the most successful cases. The contamination found in fractions from other sources falls in the range of 1–4%. It is easy to obtain the heads from human sperm since the tails are entirely dispersed by the vibrations; however, a small part of the midpiece tends to remain with the head. The sonic treatment resulted in a loss of the acrosome from only the guinea pig sperm.

Claude (1942) succeeded in separating granules of melanin from the liver of *Amphiuma tridactylum* by alternate centrifugations of 3 min. at 18,000 times gravity and 1 min. at 1500 times gravity. The granules were elongated, having dimensions of approximately $1 \times 0.5\ \mu$. A small proportion of melanin granules was found in guinea pig liver, and they bore a striking resemblance to those separated from the amphibian liver.

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